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TARGETING THE IL-33/ST2 PATHWAY IN ASTHMA

-IMPLICATIONS FOR DEVELOPMENT, EXACERBATION AND TREATMENT-

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TARGETING THE IL-33/ST2 PATHWAY IN ASTHMA

-Implications for development, exacerbation and treatment-

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Till min familj

" Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning"

Winston Churchill

ABSTRACT

Asthma is a term that encompasses a disease spectrum with different phenotypes that vary in severity and whose common characteristic is airflow limitation. Allergic asthma is the most common phenotype of asthma and is characterized by allergen induced inflammatory responses, airway hyperresponsiveness (AHR), and remodeling. The limitations of current treatment together with the prospect of curative therapies set the incentive for identifying novel targets for asthma treatment. The epithelium-derived cytokine interleukin-33 (IL-33) and its receptor ST2 are implicated in the initiation and progression of asthma by several genetic and experimental studies. This thesis investigates the role of the IL-33/ST2 pathway in asthma development, exacerbation and treatment, using mouse models of asthma.

In **paper I**, we investigated the role of IL-33/ST2 signaling in promoting allergen-induced AHR, airway inflammation, and remodeling in a mouse model of asthma, in which wild-type and $ST2^{-/-}$ mice were exposed to intranasal instillations of house dust mite (HDM) extract. We revealed that ST2-dependent signaling is important for the development of AHR in the peripheral lung compartment and for the development of inflammatory responses including airway eosinophilia, induction of allergen-specific IgE, inflammation and goblet cell hyperplasia in the peripheral airways, and production of the cytokines IL-5, IL-13 and IL-33.

In **paper II**, we tested the hypothesis that IL-33/ST2-dependent mast cell responses contribute to the development of AHR and airway inflammation, using a model where the lungs of mast cell deficient mice were engrafted with either wild-type or $ST2^{-/-}$ bone marrow derived mast cells, and subsequently exposed to HDM. Unexpectedly, we discovered a protective role for ST2-dependent mast cell responses in the development of AHR located in the peripheral lung. This protective effect appeared to be independent of airway inflammation but was associated with elevated levels of PGE_2, which has a bronchoprotective role in asthma.

In **paper III**, we explored the potential of IL-33 to exacerbate allergen-induced asthma responses by exposing OVA-sensitized mice to IL-33 before each antigen challenge. We found that IL-33 acted cooperatively with antigen to aggravate AHR, remodeling and several inflammatory responses, including a substantial potentiation of antigen-specific IgE antibody production, increased mast cell activity, elevated levels of the T_H2 cytokines IL-4, IL-5 and IL-13, and accumulation of inflammatory cells in the airways and lung tissue, including expansion of the ILC2 population in the lungs.

In **paper IV**, we evaluated the effects of vaccination against IL-33 in a mouse model of HDM-induced asthma. The vaccine comprised a recombinant IL-33 protein modified to induce immunological memory response, while reducing its cytokine activity. Vaccination against IL-33 induced high titers of anti-IL-33 IgG antibodies, and attenuated several HDM-induced responses including AHR, airway eosinophilia, accumulation of inflammatory cells in the airways, and the levels of inflammatory cytokines including IL-25, IL-33 and TSLP.

In conclusion, the work presented in this thesis provides further evidence and new insights into the importance of the IL-33/ST2 pathway in the development of asthma. The studies of this thesis identify an important role for this pathway in the regulation of AHR in the peripheral lung compartment, a protective role on AHR mediated by mast cells, and a role in asthma exacerbations associated with the expansion of the ILC2 population. Finally, we demonstrate that targeting the IL-33/ST2 pathway by vaccination against IL-33 has the potential to be an effective therapeutic tool for treating asthma.

LIST OF SCIENTIFIC PAPERS

I. Anna Zoltowska, Ying Lei, Barbara Fuchs, Carola Rask, Mikael Adner and Gunnar Nilsson
The interleukin-33 receptor ST2 is important for the development of peripheral airway hyperresponsiveness and inflammation in a house dust mite mouse model of asthma.
Clinical & Experimental Allergy 2015; 46, 479-490

II. Anna Zoltowska Nilsson, Ying Lei, Mikael Adner and Gunnar Nilsson
Mast cell dependent IL-33/ST2 signaling is protective against the development of airway hyperresponsiveness in a house dust mite mouse model of asthma
Manuscript

III. Lisa Sjöberg*, Anna Zoltowska Nilsson*, Ying Lei, Joshua Gregory, Mikael Adner and Gunnar Nilsson
Interleukin 33 exacerbates antigen driven airway hyperresponsiveness, inflammation and remodeling in a mouse model of asthma
Accepted Scientific Reports 2017

*These authors contributed equally

IV. Ying Lei, Vamsi Boinapally*, Anna Zoltowska*, Mikael Adner, Lars Hellman and Gunnar Nilsson.
Vaccination against IL-33 Inhibits Airway Hyperresponsiveness and Inflammation in a House Dust Mite Model of Asthma
PLoS One. 2015; 10(7): e0133774

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CONTENTS

LIST OF ABBREVIATIONS

AHR	Airway hyperresponsiveness
ASM	Airway smooth muscle
APC	Antigen presenting cell
BALF	Bronchoalveolar lavage fluid
BMMC	Bone marrow-derived mast cells
Col1a1	Collagen, type I, alpha I
Col3a1	Collagen, type III, alpha I
Col5a1	Collagen, type V, alpha I
DC	Dendritic cell
ELISA	Enzyme linked immunosorbent assay
FEV_1	Forced expiratory volume in 1 second
FOT	Forced oscillation technique
HDM	House dust mite
G	Tissue damping
H	Tissue elastance
Ig	Immunoglobulin
IL	Interleukin
IL1RL1	Interleukin 1 receptor-like 1
ILC2	Innate lymphoid cell group 2
MCh	Metacholine
MHC	Major histocompatibility complex
i.n.	Intranasal
i.p.	Intraperitoneal
i.v.	Intravenous
OVA	Ovalbumin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PRR	Pattern recognition receptor
R_N	Newtonian resistance
T_H	T helper
TLR	Toll like receptor
TSLP	Thymic stromal lymphopoietin

1 INTRODUCTION

1.1 ASTHMA

Asthma is a common disease of the lungs affecting more than 300 million people worldwide and is associated with substantial morbidity and economic burden [1]. The definition of asthma is controversial but the condition is usually described as a chronic disorder of the airways that is characterized by variable symptoms of reversible airflow obstruction, airway hyperresponsiveness (AHR) and underlying inflammation. The clinical presentation varies depending on severity, but manifests usually as recurrent episodes of wheezing, chest tightness, shortness of breath and cough [2]. These episodes can be triggered by exposures to various environmental stimuli such as allergens, and infectious agents, particularly viruses.

Traditionally, two major forms of asthma have been defined in the clinic; allergic asthma, which usually has an early onset, and non-allergic (intrinsic) asthma that often manifests later in life. Most children and nearly half of adults with asthma have allergic asthma that coincides with allergic sensitization characterized by the presence of T_H2 inflammation. However, it is increasingly recognized that asthma is not a single disease but rather an umbrella term encompassing a spectrum of diseases, where the same observable characteristic (phenotype) can arise as a consequence of different underlying cellular and molecular mechanisms (endotypes) [2, 3]. This has in turn awakened an interest for identifying pathophysiological mechanisms and biomarkers that distinguish subsets of patients based on various aspects including asthma symptoms, relative involvement of the large and small airways, innate and adaptive immune responses to allergens, susceptibility to viral infections leading to exacerbations, and response to treatment.

In general, targeting components of T_H2 inflammation in allergic asthma with selective therapies, including targeting of individual T_H2 cytokines, has so far been disappointing and therapy still mostly relies on bronchodilators and corticosteroids that do not affect the underlying dysregulated immune response. In light of this, a new case is made that asthma originates in the airways themselves and involves defective structural and functional behavior of the epithelium in response to environmental insults that creates a chronic wound scenario involving tissue injury and impaired repair [4]. Specifically, it has been proposed that aberrant interactions between epithelial cells and underlying resident cells are fundamental for the development of asthma [4, 5]. In this setting there is a particular interest in the epithelium-derived cytokine IL-33 and its receptor ST2, mainly due to the strong association to asthma risk in genome wide association studies [6-8]. The relevance of this association has been highlighted in several studies in asthma patients and experimental models [9-11]. Together, these finding implicate the IL-33/ST2 pathway as a promising target for novel treatment strategies.

This thesis investigates the role of the IL-33/ST2 pathway in asthma development, exacerbation and treatment. More specifically, modulation of this pathway is investigated in the context of allergen-induced asthma in mouse models where the cardinal features of asthma including airway inflammation, AHR and remodeling are evaluated.

The profile of airway inflammation varies depending on the asthma subtype. The most common inflammatory profile of asthma is associated with eosinophilia and type 2 inflammation. However, some patients show a neutrophil-predominant inflammation with an absence of T_H2 cytokines. This type of inflammation is characteristic of patients with late-onset and more severe forms of asthma with less reversible airway obstruction and a mixed T_H1 and T_H17 cytokine environment [12-14].

1.1.1 Type 2 inflammation in asthma

Type 2 immune responses can be highly appropriate when initiated by for instance helminth infections that lead to functional alterations that favor worm expulsion. On the other hand, misdirected type 2 responses to harmless allergens can have detrimental effects leading to atopic diseases, such as allergy and asthma. Type 2 inflammatory responses in the lungs often start in childhood. It is believed that exposure to environmental agents such as respiratory virus infections and allergens can initiate pathogenic immune responses, which lead to the development of asthma in children who are predisposed because of specific genetic risk factors in important regulators of type 2 inflammation (atopy), or because of other vulnerabilities [15].

The type 2 cytokines, including IL-4, IL-5 and IL-13, are highly associated to the pathogenesis of asthma, regulating many key responses, including eosinophilia, IgE synthesis, bronchoconstriction, and mucus production [16-19]. More specifically, IL-4 is involved in the isotype class switching of B cells to the synthesis of IgE antibodies, which can be bound to high affinity Fcε receptors (FcεRI) on mast cells and basophils [20]. IL-5 activates and recruits eosinophils to the lung, where they secrete inflammatory cytokines and chemokines [21]. IL-13 can directly affect the airway epithelium and smooth muscle (ASM) cells and thereby contribute to AHR, mucus hyperproduction, and in chronic inflammation, to airway remodeling [22, 23]. T_H2 cells are key players in allergic asthma, as they are major producers of these prototypical cytokines [24, 25]. In addition to T_H2 cells, it is now recognized that group 2 innate lymphoid cells (ILC2s) are an important and earlier source of T_H2 cytokines, particularly IL-5 and IL-13 [26]. Mast cells are also an important source of type 2 cytokines including IL-4, IL-5 and IL-13 [27, 28]. ILC2s seem to secrete very little IL-4 and the relative importance of T_H2 cells, ILC2s, and mast cells in the production of type 2 cytokines is still unclear [29]. Nevertheless, there seems to be a consensus that upstream events in the airway epithelium, involving epithelium derived key regulatory cytokines, such as IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), initiate both adaptive and innate type 2 responses that lead to increased production of type 2 cytokines [15]. IL-33 regulation of these events are further discussed in section 1.6.5 where the current knowledge about IL-33 from experimental studies are put in the context of type 2 responses identifying potential mechanisms by which IL-33 contributes to development of asthma. Central to this thesis are the highly IL-33 responsive ILC2s and mast cells, which are described in general in the following sections and further in the context of IL-33 responses in section 1.6.5.

1.1.1.1 ILC2s

ILCs are tissue-resident innate cells, which are classified into three different subtypes; ILC1s, ILC2s and ILC3s, based on a similar cytokine profile corresponding to the helper T subsets T_H1, T_H2, and T_H17, respectively [29]. They are promptly activated by danger signals from injured mucosa and thereby maintain mucosal integrity in response to invading pathogens. It is becoming increasingly recognized that the phenotype of ILCs is not stable. Indeed, ILCs are highly plastic and can change phenotypes under the influence of environmental triggers [30].

The observation that RAG-/- mice, lacking mature T cells and B cells, were still able to mount a strong type 2 response challenged the role for T_H2 cells in driving type 2 responses [31, 32]. This finding suggested that there were pathways independent of the adaptive immune system that could induce type 2 responses and led to the identification of a previously unrecognized innate cell population, currently termed type 2 lymphoid cells (ILC2s) [33]. ILC2s resemble T_H2 cells in their capacity to produce type 2 associated cytokines but due to the absence of rearranged antigen-specific receptors, their response is not antigen-specific [26]. Thus, instead of becoming activated by antigen presentation through antigen-presenting cells (APCs), ILC2s respond by sensing danger signals from the tissue environment.

Several cytokines can activate ILC2s including the epithelium derived cytokines IL-25, IL-33, and TSLP, which have in particular been shown to initiate ILC2 responses [34]. The relative importance of these cytokines in activating ILC2s seems to vary depending on the experimental setting. Airway challenge of IL-33 receptor deficient ($ST2^{-/-}$) mice with ragweed pollen or ovalbumin suggests that IL-33 is more potent than IL-25 in promoting pulmonary ILC2 activation [35]. TSLP was shown to synergize with IL-33 to produce IL-5 and IL-13 in a chitin induced model of airway inflammation in mice [36], and ILC2 activation was only abolished in the combined absence of IL-25, IL-33, and TSLP signaling [37]. Recently, it was reported that mast cell derived IL-2 leads to the expansion of ILC2s in a positive feedback loop that is driven by IL-9, of relevance in driving inflammation in patients with cystic fibrosis [38]. In the proposed model, IL-9 produced by IL-33-expanded ILC2s, activates mast cells for IL-2 production leading to the expansion of ILC2s that in turn promote T_H9 activation, and the resulting IL-9 production further amplifies the inflammatory loop. Additionally, ILC2s have been shown to be activated by leukotrienes [39] and prostaglandins [40, 41].

Mouse models of asthma that use ovalbumin (OVA), house dust mite (HDM), fungal allergens, and papain, show that ILC2s increase in number and are the major source of IL-5 and/or IL-13, particularly in the early phase [26, 42, 43]. Allergens can induce expansion of ILC2s, high levels of type 2 cytokines, and significant AHR in mice lacking an adaptive immune system [42, 44].

The role of ILC2s in the airways of patients with allergic asthma has been investigated by several studies. An increased frequency of IL-13 producing ILC2s was found in the BALF from asthma patients compared to control subjects [45]. Higher levels of ILC2s were

observed in sputum of patients with corticosteroid resistant severe asthma compared to healthy controls as well as with patients with mild asthma [46]. An increased number of ILC2s was also found in sputum of children with severe therapy-resistant asthma compared with children without asthma but with lower respiratory tract infections [47].

1.1.1.2 Mast cells

Mast cells originate from pluripotent hematopoietic stem cells, which circulate as $CD34^+$ precursors in the blood before migrating into tissues where they mature into long-lived effector cells [48-50]. As tissue resident cells, mast cells predominantly populate tissues facing the external environment such as the skin, intestine and lung [51]. Mast cells are often located in close proximity to blood and lymph vessel, and nerves where they can release locally or systemically active mediators [52]. In humans, mast cell subtypes are divided based on their protease content. MC_T store tryptase, while MC_{CT} store both chymase and tryptase in their granula [53]. MC_T are predominantly found in the lung and intestinal mucosa, whereas MC_{CT} appear in the skin and lymph nodes. However, this division is likely an oversimplification, as site-specific microenvironment dependent subpopulations of these phenotypes exist in the lung [54]. Two types of mast cells are recognized in mice; mucosal mast cells (MMCs) and connective tissue mast cells (CTMCs) [55-58]. Mast cell mediators are either stored in granules, and can be released immediately during degranulation, or synthesized *de novo* after activation and are released within minutes to hours after activation [59, 60]. Accordingly, mast cell mediators can be divided into three groups; 1) preformed substances, 2) lipid mediators and 3) cytokines and chemokines [49].

Mast cells express a variety of receptors associated with innate and adaptive immunity. There are a wide range of stimuli that can modulate mast cell responses, including IgE-mediated crosslinking of the membrane bound IgE high affinity receptor (FcεRI), which is the most well characterized pathway of mast cell activation [61]. Activation of mast cells can also occur by specific microbial patterns via Toll like receptors (TLRs) [62, 63], and by the epithelial derived cytokines IL-33 and TSLP, via the ST2 and TSLP receptor, respectively [64, 65].

Mast cells and several mast cell derived mediators have been shown to be important during the development of allergic airway disease. They are primarily located adjacent to blood vessels in the lamina propria of airway mucosa in human airways. In patients with asthma mast cells can also be found in proximity to other key structures that are involved in the pathophysiology of asthma including the airway epithelium, mucous glands, and airway smooth muscle [27, 66, 67]. There exists an extensive crosstalk between mast cells and smooth muscle cells as evident from *in vitro* studies of their interactions. Mast cells have been shown to induce differentiation of smooth muscle cells into a more contractile phenotype [68]. In turn, smooth muscle cells can enhance mast cell survival and induce degranulation [69].

Increased concentrations of mast cell mediators such as histamine, leukotrienes, proteases and prostaglandins can be found in the BALF of aeroallergen challenged patients with allergic asthma [70-72]. The rapid release of mast cell mediators by allergen crosslinking of

membrane bound IgE results in the early allergic reaction that happens within minutes and causes contraction of the airway smooth muscle (bronchoconstriction), vasodilation, increased airway permeability and epithelial mucus production leading to airflow obstructions. Additionally, these mediators also lead to the late phase response that is characterized by inflammatory cell infiltration (by eosinophils, $CD4^+$ T cells, neutrophils, mast cells and basophils), and associated with bronchial swelling and AHR. In support of the importance of mast cells in these reactions, treatment with anti-IgE antibodies, and histamine and leukotriene receptor antagonist, completely inhibits the development of the early phase response and in part also the late phase response [73-75].

1.2 AIRWAY HYPERRESPONSIVENESS

Airway hyperresponsiveness (AHR) is a consistent and cardinal feature of asthma and is defined by an exaggerated response of the airways to narrow or close in response to stimuli that would produce little or no effect in healthy individuals [76]. The presence of AHR is associated with an increased decline in lung function and the severity with an increased risk of exacerbation [77, 78].

1.2.1 Measurement of AHR

AHR is usually measured using bronchial provocation tests during which a bronchoconstrictive agent is inhaled in increasing doses, which assists in making clinical diagnosis of asthma. Spirometry is performed before and after each dose, and a 20% fall in FEV_1 (forced expiratory volume in one second) is considered a positive reaction and the provocative dose that causes this reaction (the PD20) is used to indicate the level of airway hyperresponsiveness [76]. The test is considered negative if FEV_1 does not fall by at least 20% with the highest concentration. There are two main groups of bronchoconstrictive agents used to measure AHR; those which are thought to act directly on the airway smooth muscle receptors to induce bronchoconstriction and those with an indirect bronchoconstrictive effect that is mediated by the release of mediators following the primary stimuli [79]. Direct challenge tests include agents such as methacholine or histamine and indirect challenge tests include mannitol, various allergens and exercise. The traditional determination of AHR using spirometric parameters like FEV_1 is highly effort dependent, which may pose a problem in evaluating some patients including small children as the method requires substantial co-operation. In contrast, the forced oscillation technique (FOT) is a measure of respiratory mechanics that can be acquired employing small-amplitude pressure oscillations superimposed on the normal breathing, and therefore is not dependent on performance of breathing manoeuvers.

1.2.2 Mechanisms of AHR

The mechanisms underlying AHR in asthma is still poorly understood, most likely different mechanisms or a combination of these give rise to AHR in different patients [80]. AHR can be seen as consisting of two components; the acute and variable AHR, which is closely associated with an episodic increase in airway inflammation due to environmental factors such as allergen exposure, and a baseline AHR, which is persistent and presumably caused by

airway remodeling due to chronic airway inflammation [81]. Although, airway inflammation, structural remodeling and functional changes in the airway smooth muscle likely contribute to AHR, the mechanisms that link these changes are not clear.

There is a positive correlation between the severity of AHR and the number of eosinophils in sputum [82] , as well as mast cells in the airways of patients with asthma [67], which suggests that these factors may contribute to the severity of AHR. Since bronchoconstriction is mediated by airway smooth muscle (ASM) surrounding the airway, the contractility of ASM has been implicated as a principal cause of AHR. In support of this notion, a study employing gene expression profiling of ASM revealed several genes that differentiated asthmatic and non-asthmatic patients and related to the severity of AHR [83]. Moreover, the expression of contractile proteins such as α-smooth muscle actin in ASM from asthma patients correlates with AHR severity [84]. ASM responsiveness seems to be regulated by several pro-inflammatory mediators including IL-4, IL-13 and tumor necrosis factor-α (TNF-α), potentially through effects on calcium signaling [85]. Interestingly, the number of mast cells within the ASM correlates with AHR severity in asthma [86, 87] and mast cell mediators such as histamine and prostaglandin D_2 (PGD_2) may contribute to increased basal tone of the ASM [88, 89]. Furthermore, AHR correlates with several changes related to structural airway remodeling [90-92].

1.3 AIRWAY REMODELING

Airway remodeling in asthma refers to the pathological changes that occur in the airway epithelium and submucosa which are associated with an irreversible loss of function that tracks from childhood to adulthood [93]. Epithelial changes include goblet cell metaplasia, hyperplasia and increased mucin accumulation. Submucosal changes include subepithelial fibrosis (characterized by increased deposition of collagen type I, III and V beneath the basement membrane), increased volume of submucosal gland cells, smooth muscle cell hypertrophy and hyperplasia and increased number of blood vessels [94-96]. These structural changes are considered to predispose to asthma exacerbations due to narrowed airways at baseline and increased sensitivity to inhaled exacerbants such as viruses or allergens [97].

The relationship between airway inflammation, remodeling and AHR remains controversial. Human and animal studies have for a long time supported the hypothesis that chronic inflammation may drive remodeling in patients with asthma. This theory has mainly been supported by the finding that steroid treatment in patients with asthma in addition to reducing airway inflammation also has beneficial effects on airway remodeling [92, 98, 99]. Although the classical type 2 cytokines, IL-4, IL-5, IL-9 and IL-13, have prominent functions in directing the production of allergen-specific IgE, recruitment of eosinophils or development of AHR; their impact on airway remodeling is still unclear. In fact, IL-13 seems to be the only type 2 cytokine with profound effects on airway structural cells, as it induces mucin expression and mucus metaplasia in both epithelial cells and submucosal glands, and also has a crucial role in goblet cell hyperplasia and metaplasia [100, 101].

However, an increasing amount of studies suggest that airway remodeling, rather than a secondary event to inflammation, can evolve as a primary event initiated early in life in the

absence of any apparent inflammation [102]. In support of this notion, several studies show that already preschool children have evidence of remodeling, including a study where airway remodeling manifested in preschool children with severe wheeze, without any apparent relationship between airway remodeling and inflammatory cell counts in biopsies [103]. Furthermore, a study using an HDM-induced mouse model of early-onset airway disease, demonstrated that inflammation, remodeling and AHR develop in parallel rather than sequentially [104]. Experimental models also suggest a closer relationship between airway contraction, lung function and remodeling than between inflammation and remodeling, and show that remodeling can develop independently of inflammation [105, 106].

Perhaps these contradictory results, indicating on one hand that airway remodeling is a secondary event following chronic airway inflammation, and on the other hand occurring as a primary event, are in fact yet another reflection of the heterogeneity of asthma and both could indeed hold depending on the asthma subtype.

1.4 TREATMENT

The mainstay of asthma management includes inhaled corticosteroids (ICSs) and short- and long-acting β_2-adrenoceptor agonists (SABAs and LABAs). A stepwise management approach with incremental dosing and additional controller medication is enforced with the goal to achieve symptom control and prevent exacerbations [107]. Inhaled SABAs are used as relievers of bronchoconstriction when required (step 1) together with ICSs as controllers to reduce airway inflammation (step 2). Asthma that is not well controlled by this strategy may benefit by supplementary therapy with inhaled LABAs or an increased dose of ICSs (step 3). Uncontrolled symptoms despite these efforts requires additional intervention in the form a forth drug such as a leukotriene receptor antagonists (LTRAs) or a further dose increase of inhaled corticosteroids (step 4) and as a final resort treatment with daily oral corticosteroids might be required (step 5).

Conventional asthma treatments are continuously updated for new molecules as well as more effective and convenient delivery devices. The major drawbacks with the usage of ICS, LABAs, and oral corticosteroids are the risk of long-term side effects and relapse of symptoms after discontinuing the treatment. Furthermore, a considerable number of patients remain refractory despite oral corticosteroids. Even novel therapeutics that have achieved improvements in disease control by targeting type 2 mediators, predominantly consisting of monoclonal antibodies directed to IgE [108], IL-5 [109] , IL-13 [110], or IL-4[111] are only applicable to a subgroup of patients and do not affect the underlying dysregulated immune response [112].

The only disease modulating approach for allergic asthma is allergen-specific immunotherapy (AIT). However, specific immunotherapy by subcutaneous injection of allergen (SCIT) is not very effective in controlling asthma and is associated with serious side effects, particularly in patients with uncontrolled and severe asthma [112, 113]. Sublingual immunotherapy (SLIT) with HDM extracts has shown some efficacy in asthma and seems to be well tolerated, but is limited to a subgroup of patients and needs to be evaluated in comparison to ICSs in long-term studies [114].

Considering the limitations of available treatment strategies for asthma, including the considerable number of patients that remain uncontrolled, there is a need to identify underlying mechanisms and novel targets with the prospect of developing curative or preventive therapies for asthma.

1.5 ASTHMA EXACERBATIONS

Asthma exacerbations are defined as an exaggerated lower airway response to an environmental exposure [115]. When exacerbations of asthma occur, the underlying clinical features of asthma are accentuated with a subsequent deterioration of asthma control, often despite of treatment [116, 117]. Respiratory viral infections are the leading cause of asthma exacerbations, associated with nearly 80% of asthma exacerbation episodes [118, 119]. In the majority of patients with asthma, exacerbations are caused by comparably mild respiratory viruses, including human rhinovirus (HRV), respiratory syncytial virus (RSV), or by influenza virus [116]. Allergen exposure is another common cause of asthma exacerbations and more than 80% of children with asthma are sensitized to environmental allergens [117]. Common airborne allergens include house dust mites, molds, animal dander, weeds, trees and grass pollen [117].

Although asthma is well-controlled in the majority of patients, a significant proportion of patients experience exacerbations, which are the leading cause of hospital admission in industrial countries among children with asthma [120]. Respiratory virus infections are major risk factors for hospital admissions particularly in combination with allergen exposure in sensitized children acting in synergy to increase the risk of hospital admission [121, 122]. There are currently no vaccines available for the viruses that are the leading cause of asthma exacerbations. Allergen-specific immunotherapy is generally not recommended for patients with uncontrolled asthma [112, 113]. Although, monoclonal anti-IgE antibodies (omalizumab) can reduce asthma exacerbations, its use is limited to the most severe cases because of high cost and requirement for regular injections. In conclusion, there is a need for improved therapeutic strategies in the prevention and management of asthma exacerbations. Understanding the mechanisms by which viral infections and allergen exposure exacerbate the pathophysiology of asthma is an important step towards improved treatment.

1.6 THE IL-33/ST2 PATHWAY AND ASTHMA

IL-33 is implicated as an asthma susceptibility gene through genome wide association studies (GWAS) and its relevance is supported by several findings in asthma patients as well as experimental mouse models of asthma.

1.6.1 Genetic association of the IL-33/ST2 pathway and asthma

Several genetic studies have linked variants in the genes for IL-33 (*IL33*) and its receptor ST2 (*IL1RL1*) with asthma risk. This association is well established and has been replicated in different populations [8, 123, 124], in early childhood asthma [6], as well as in severe forms asthma in adults[8]. Initially, a genome wide association study (GWAS) identified single nucleotide polymorphisms (SNPs) in *IL33* to be associated with elevated blood eosinophil counts, which led to a large follow up case-control study of asthma patients and control

subjects that indicated an association of *IL33* SNPs with asthma [125]. Subsequently, a consortium-based GWAS of over 10, 000 asthma patients identified *IL33* as one of the top hits for asthma. This study was later combined with a meta-analysis study, confirming the association between *IL33* and asthma [8]. Recently, an *IL33* loss-of-function mutation that reduces blood eosinophil counts and protects from asthma was identified in an Icelandic population [126].

1.6.2 The IL-33/ST2 pathway and findings from asthma patients

Several studies in asthma patients have highlighted the relevance of the genetic association between the IL-33/ST2-axis and asthma. IL-33 has been found to be increased in the bronchoalveolar lavage fluid, airway epithelial cells, and airway smooth muscle cells of patients with asthma compared with healthy controls, with a positive correlation to asthma severity [10, 45, 127, 128]. In addition, IL-33 has been shown to induce increased levels of IL-4, IL-5 and IL-13 in matured and activated cells, including mast cells, basophils and eosinophils, derived from allergic subjects compared to those from healthy controls [129, 130]. There is also a link between IL-33 levels and airway remodeling, as IL-33 expression is increased in endobronchial biopsies derived from pediatric patients with severe therapy-resistant asthma and correlates with reticular basement membrane thickening [11].

1.6.3 IL-33 and experimental findings from mouse models of asthma

Evidence for a functionally important role for IL-33/ST2 in asthma also comes from several experimental findings in mouse models. Intranasal administration of IL-33 has been shown to induce airway inflammation, promote AHR, goblet cell hyperplasia, eosinophilia as well as accumulation of lung IL-4, IL-5 and IL-13 [127, 131-133].

1.6.4 Molecular aspects of IL-33

IL-33 is a member of the IL-1 family of cytokines and was first identified as a nuclear factor expressed in lymph node-associated endothelial cells and was later identified as the ligand for the former orphan receptor ST2 [134]. Unique within the IL-1 family, IL-33 is associated with the promotion of type 2 immune responses.

1.6.4.1 IL-33 production and release

IL-33 is constitutively expressed in cell types involved in maintaining mechanical barriers including keratinocytes and epithelial cells in lung and gut, as well as endothelial cells, fibroblasts and smooth muscle cells [134-136]. Recently, the main source of IL-33 in response to chitin was identified to be alveolar type II cells in the distal airways [36]. Although, IL-33 can be induced in leukocytes (including macrophages, dendritic cells, mast cells and monocytes) during inflammation [137-139], their relative importance as an IL-33 source is less clear as their IL-33 expression levels are ten-fold lower relative to epithelial cells.

Upon synthesis, IL-33 translocates from the cytosol to the nucleus, where it associates with chromatin and can regulate gene expression by several mechanisms such as the

downregulation of soluble ST2 and IL-6 expression, and sequestering of the nuclear factor-κB (NF-κB) [136, 140-145]. During cell death by injury or necrosis, the stored full-length IL-33 in the nucleus is passively released and in this context IL-33 has been classified as a damage-associated molecular pattern (DAMP) or an alarmin [146]. The full-length IL-33 released under necrotic conditions is biologically active, while it is inactivated by caspase-3 and caspase-7 during apoptosis [147]. In addition to the passive release of IL-33, a yet unknown mechanism for controlled IL-33 release must exist, as stimulation of different cell types results in release of bioactive IL-33 in the absence of cell injury and necrosis. Cardiac endothelial cells and fibroblasts subjected to mechanical stress release IL-33 without cell death [148, 149]. Exposure to HDM with proteolytic activity has been shown to trigger TLR4 on the pulmonary epithelial cells resulting in the production of IL-33 [150]. Similarly, exposure of human epithelial cells to the fungus *Alternaria alternata* induced rapid release of IL-33 via ATP signaling [151]. However, the molecular mechanism by which IL-33 is released is enigmatic as IL-33 lacks a signal sequence that can enable classical protein secretion through the endoplasmic reticulum-Golgi pathway [152]. It has been suggested that IL-33 release can be mediated by several non-classical pathways involving different types of secretory vesicles [153].

1.6.4.2 IL-33 signaling

Once outside the cell, IL-33 acts as a classical cytokine exerting its activity by binding to a transmembrane heterodimer formed by its specific primary receptor ST2 (also known as IL1RL1, T1 or IL1R4) [134]. ST2 undergoes a conformational change after ligand binding, which allows for the recruitment of the co-receptor IL-1RAcP (also known as IL-1R3), resulting in the formation of the minimal heterodimer complex IL-33R [154]. More sophisticated forms of the IL-33R exist in some cell types. IL-33 signaling in mast cells can be synergistically amplified by the crosstalk between IL-1RAcP and the activated KIT receptor [155].

Ligand binding and the subsequent formation of the IL-33R complex initiates signal transduction in the target cell by the MyD88-IRAK-TRAF6 signal module [156], which is proposed to activate at least two pathways including NF-kB and mitogen-activated protein MAP kinases (MAPKs) [134].

1.6.4.3 Mechanisms modulating IL-33 activity

Nuclear localization or retention of IL-33 is essential for limiting the inflammatory effects of IL-33 and thereby for maintaining immune homeostasis [157]. Following the release of IL-33, several mechanisms could limit the bioactivity of the full-length IL-33. Soluble forms of ST2 (sST2) and IL-1RAcP exist, which have been proposed to function as decoy receptors to neutralize IL-33 [148, 149]. Moreover, inflammatory proteases derived from different cellular sources, including mast cells and neutrophils, are able to modulate IL-33 activity by cleaving the full-length protein at the N-terminal domain, thereby increasing the biological activity several-fold [158, 159]. Thus, the potency of IL-33 can be quickly increased at the site of inflammation. In contrast, disruption of the C-terminal core results in the loss of IL-33 bioactivity, as illustrated by the potential to limit IL-33 signaling by mast cell chymase [160,

161]. In addition to proteolytic cleavage, inactivation of extracellular IL-33 can be accomplished by oxidation of cysteine residues and the formation of disulfide bridges that prevent ST2 binding [162].

1.6.4.4 ST2 expression

The expression of ST2 is widespread, but transmembrane ST2 was initially shown to be most highly expressed on mast cells and to a lesser extent on T_H2 cells, which led to the association between the IL-33/ST2 signaling pathway and the type 2 mediated diseases such as asthma [124, 163]. Other $ST2^+$ cells include ILC2s, Treg cells, dendritic cells (DCs), macrophages, basophils and eosinophils [164].

1.6.5 IL-33 regulation of type 2 immune responses in asthma

IL-33 interacting with ST2 on a range of different leukocytes promotes a number of key inflammatory pathways of both innate and adaptive immunity that are involved in the initiation and propagation of allergen driven type 2 responses. This section aims to describe the current knowledge of IL-33 functions, from both *in vivo* and *in vitro* studies, in the context of allergen driven type 2 immune responses in asthma (Fig. 1).

Following exposure to airborne allergens or respiratory viruses, IL-33 is released from the pulmonary epithelium either passively, as a result of cell damage, and/or actively by triggering epithelial cell activation through pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) [165]. Interestingly, exposure to HDM allergen has been shown to induce the release of IL-33 as a result of TLR4 triggering on the pulmonary epithelial cells [150]. Exposure to the fungus *Alternaria* in mice was reported to induce a rapid release of IL-33 to the BALF [166]. Similarly, exposure of human epithelial cells to *Alternaria* induced rapid release of IL-33 via ATP signaling [151]. Furthermore, IL-33 can be released as a result of viral infection as shown in studies in mouse ILCs infected with influenza virus [167].

Released IL-33 can likely promote allergen driven responses by effects on $ST2^+$ innate and adaptive immune cells that promote allergen sensitization and type 2 pathology via mainly generation of IL-4, IL-5 and IL-13 [22, 168]. In a naïve situation the primary tissue resident cells including ILC2s, mast cells, DCs, and alveolar macrophages, with a constitutive expression of ST2 constitute the initial targets of IL-33 [169, 170]. IL-33 is an important activator of ILC2s, which provide an important and early source of IL-5 and IL-13 [26]. Direct actions of IL-33 on mast cells include their promotion of maturation, activation, survival and adhesion [65, 171-173]. IL-33 has a profound effect on the phenotype of alveolar macrophages, driving them towards an alternatively activated (AAM) phenotype that contributes to innate and antigen-induced airway inflammation [127].

Airway DCs play a crucial role as antigen presenting cells (APCs) in initiating the adaptive arm of type 2 responses in asthma. Dendritic cells located at the airway lumen sample and take up antigens and, after becoming activated, migrate to the draining lymph nodes where they present processed antigenic peptides on MHC class II molecules to naïve $CD4^+$ T cells [174]. This mechanism defines the onset of immunological memory, which involves the

activation of $CD4^+$ T cells to become IL-4 competent [15]. IL-33 has been shown to activate DCs by the induction of OX40 ligand (OX40L) expression that promotes the mobilization of DCs to local draining lymph nodes [15, 175].

The IL-4 competent $CD4^+$ T cells migrate to B cell zones where they differentiate into T follicular (T_{FH}) helper cells or enter the circulation to mature into T_H2 cells. T_{FH} cells are able to induce IgE class-switching in B cells via IL-4 in the B cell follicular area, while T_H2 cells can migrate to the airway epithelium and mediate their action through the secretion of cytokines such as IL-5 and IL-13 [15, 22, 176]. IL-33 can also act directly on $ST2^+$ T_H2 cells to promote their recruitment via an unknown mechanism [177], and to induce production of cytokines including IL-5 and IL-13 [130, 131, 134, 178].

IgE^+ B cells produce IgE antibodies, which in turn bind to the high-affinity IgE Fc immunoglobulin receptor (FcεRI) expressed on mast cells and basophils enabling allergen-specific activation and the rapid production of vasoactive amines and lipid mediators on allergen encounter [15]. IL-33 also exerts distinct effects on basophils, enhancing their IgE-mediated degranulation, migratory capacity, and synergizes with IL-3 to promote IL-4 production and CD11b expression [22, 179-181]. Eosinophil degranulation, adhesion and enhanced eosinophil survival have also been shown to be induced by IL-33 [180-182].

Taken together, IL-33 actions on $ST2^+$ inflammatory cells has the potential to stimulate an inflammatory milieu leading to enhanced type 2 responses that include both innate and adaptive immunity that act in concert to drive the pathology of asthma including antigen-specific IgE production, eosinophilia, airway remodeling and AHR. Although many type of immune cells are $ST2^+$, and IL-33 has been shown to modulate their activity *in vitro* and in *vivo*, their relative contribution to development of asthma pathophysiology remains elusive and needs to be put in relation to other modulatory pathways.

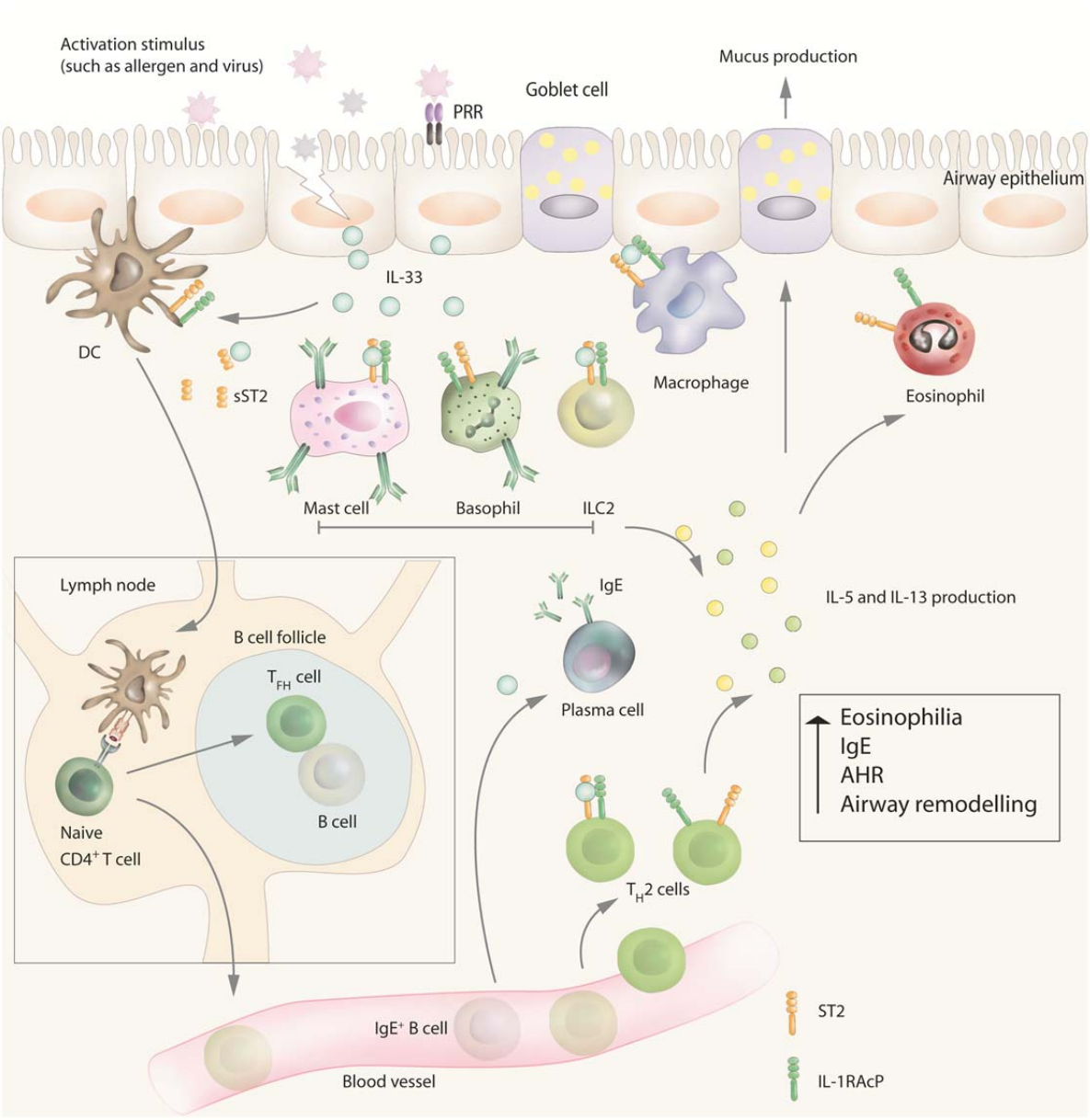

Figure 1. **IL-33 regulation of type 2 immune responses in asthma**. Following exposure to airborne allergens or respiratory viruses, IL-33 is released from the pulmonary epithelium either passively, as a result of cell damage, and/or actively by triggering epithelial cell activation through pattern recognition receptors (PRRs). IL-33 promotes the mobilization of DCs to local lymph nodes where they present processed antigenic peptides on MHC class II molecules to naïve $CD4^+$ T cells. The $CD4^+$ T cells become activated to an IL-4 competent state and migrate to B cell zones where they differentiate into T follicular helper (T_{FH}) cells or enter the circulation to mature into T_H2 cells. T_{FH} cells are then able to induce IgE class-switching in B cells via IL-4 in the B cell follicular area, while T_H2 cells can migrate to the airway epithelium and mediate their action through the secretion of cytokines such as IL-5 and IL-13. IL-33 can also activate mast cells, basophils and ILC2s to produce IL-5 and IL-13, which mediate inflammatory and remodeling changes that predispose to asthma and asthma exacerbations. (Modified from [15]).

1.7 IL-33 IN HEALTH AND DISEASE

Although IL-33 has a role in contributing to the development of allergic diseases including asthma, it is important to recognize that the role of IL-33 can be both harmful and protective depending on the disease and its context [164]. Given the pleiotropic nature of IL-33 to act on various cells of the immune system, it is not surprising that IL-33 is involved in many processes, both of protective as well as harmful nature, in host defense against pathogens [183, 184], rheumatological diseases [185, 186], cardiovascular diseases [148, 187, 188], type 2 diabetes [189] and inflammatory bowel disease (IBD) [190].

1.8 MOUSE MODELS OF ASTHMA

Mice are the most widely used species in asthma research mainly because, a wide array of specific reagents are available for analysis of cellular and mediator responses, and the availability of genetically engineered transgenic or gene-knockout mice [191]. Mouse models of asthma have been shown to be valuable tools in elucidating the cells and molecules that are responsible for pathophysiological responses in allergic airway disease [192, 193]. Although, asthma models can't serve as true representations of the disease, they able to recapitulate important aspects of the disease including airway inflammation, AHR, and in chronic protocols, remodeling [192]. Since mice do not spontaneously develop these responses, they have to be induced. Traditional protocols use systemic sensitization with antigens such as ovalbumin (OVA) with an adjuvant, followed by inhalation of the antigen during the challenge phase to induce an acute asthma-like phenotype. More recent models employ the use of more physiologically relevant allergens and sensitization routes. Commonly used allergens in these protocols include HDM extracts, *Aspergillus*, and *Alernaria alternata* that are administered via repetitive inhalation over a period up to 12 weeks [192, 194, 195].

The limitations of mouse models are mainly attributed to existing differences in lung anatomy and physiology in relation to humans. There are differences in the airway branching pattern, airway smooth muscle mass, as well as type and location of cells within human and mouse lung that can affect lung responses [196, 197]. Additionally, variability is observed within the mouse species, as there are strain-specific phenotype variations among common inbred mouse strains that exhibit considerable differences in immunological responses to the same allergen [198, 199]. The choice of strain depends on what aspect of asthma is being modelled. BALB/c mice are considered to have a more Th2-dominant immune response compared to C57BL/6 mice. However, most gene-manipulated mice are on the C57BL/6 background. Because of these differences, results obtained from studies using different mouse strains can be difficult to compare as they might reflect the difference in genetic background of the mice. Other parameters that can influence the outcome of the study include variations in sensitization and challenge protocols including the schedule and the route of administration of the allergen. In conclusion, interpolation of results from mouse to human needs to be done taking these limitations into considerations and ultimately, the findings in mouse models, need to be verified in humans.

2 THE PRESENT STUDY

2.1 AIM

This thesis investigates the role of the IL-33/ST2 pathway in asthma development, exacerbation and treatment using mouse models of allergic asthma.

Specific aims

Paper I: To investigate the importance of IL-33/ST2 signaling for the development of airway hyperresponsiveness, inflammation and remodeling.

Paper II: To examine how IL-33/ST2-dependent mast cell responses contribute to the development of airway hyperresponiveness and inflammation.

Paper III: To elucidate the role of IL-33 in promoting exacerbation of asthma in sensitized subjects.

Paper IV: To evaluate the potential of an IL-33 vaccine for preventing the development of asthma.

2.2 MATERIALS AND METHODS

The following sections provide a summary of the most important materials and methodology used in papers I-IV. Detailed descriptions can be found in the "Materials and methods" section of each paper.

2.2.1 Mice

All animal handling and experimentation was conducted in accordance with ethical permits approved by the Regional Committee of Animal Experimentation Ethics (Stockholm North ethical committee for animal welfare; Stockholm, Sweden).

Paper I Female BALB/c wild-type mice and IL-33 receptor-deficient ($ST2^{-/-}$/$Il1rl1^{-/-}$) mice on BALB/c background.

Paper II Female mast cell-deficient C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$ mice engrafted with congenic bone marrow-derived mast cells (BMMCs) from female wild-type C57BL/6 mice or IL-33 receptor-deficient ($ST2^{-/-}$/$Il1rl1^{-/-}$) mice on C57BL/6 background.

Paper III Male C57BL/6 wild-type mice.

Paper IV Female BALB/c mice.

2.2.2 Study protocols

The experimental protocols used to model allergic asthma in mice were based on exposure to HDM extract (papers I, II and IV) or OVA (paper III). Interventions introduced to these models, differences with respect to dose, routes of delivery and time intervals are outlined as follows.

Paper I Wild-type and $ST2^{-/-}$ mice were exposed to twelve intermittent intranasal instillations of HDM extract (50 μg). The outcome was assessed 48 h after the last HDM exposure (Fig. 2).

Paper II Mast cell deficient Wsh-mice were injected intravenously with *in vitro* differentiated wild-type or $ST2^{-/-}$ BMMCs. Allowing 12 weeks for engraftment, mice were subsequently subjected to the protocol described in **paper I** with the exception that a lower HDM dose was used (25 μg) (Fig. 2).

Paper III Mice were sensitized to OVA i.p. (10 μg) and subsequently challenged with OVA (200 μg) i.n. Administrations of IL-33 (0.2 μg) preceded each OVA-challenge. The outcome was evaluated 24 h after the final OVA-challenge (Fig. 3).

Paper IV Similarly to **paper I** and **paper II**, mice were subjected to the HDM-induced model of allergic asthma. The dose of HDM was the same as in **paper II** (25μg). As an intervention, mice were immunized s.c. with an IL-33 vaccine

(100 µg), with two prophylactic doses delivered prior and one dose a week after the initiation of the asthma protocol (Fig. 4).

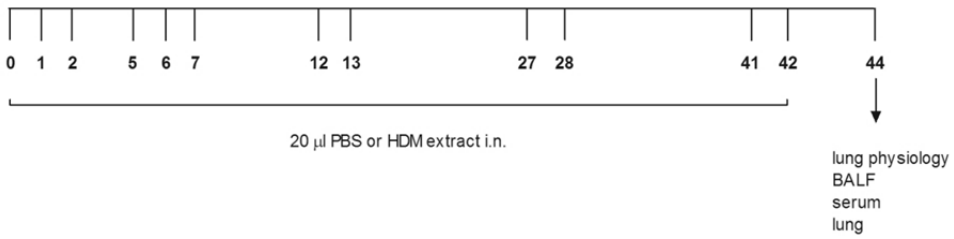

Figure 2. The experimental protocol used to induce chronic allergic asthma in **papers I, II and IV**. House dust mite (HDM) or PBS as control were administered through intranasal instillations.

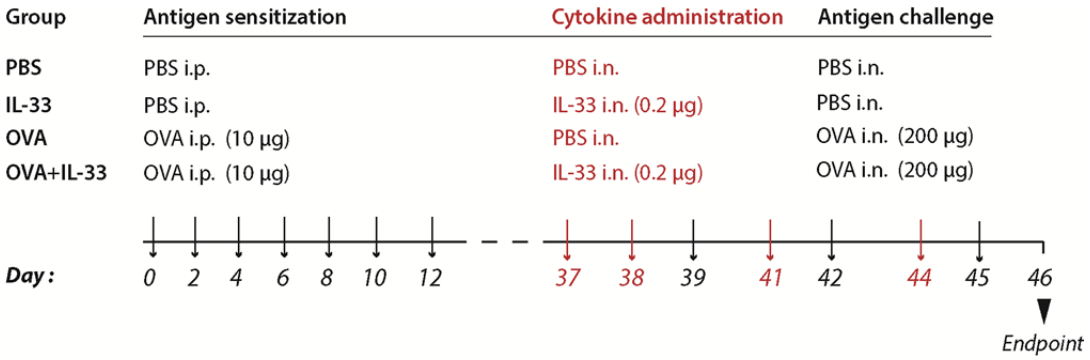

Figure 3. The experimental protocol used in **paper III**. Mice were sham- or OVA-sensitized through intraperitoneal (i.p) injections and subsequently exposed to IL-33 or PBS as control through intranasal (i.n.) instillations preceding each OVA/PBS challenge.

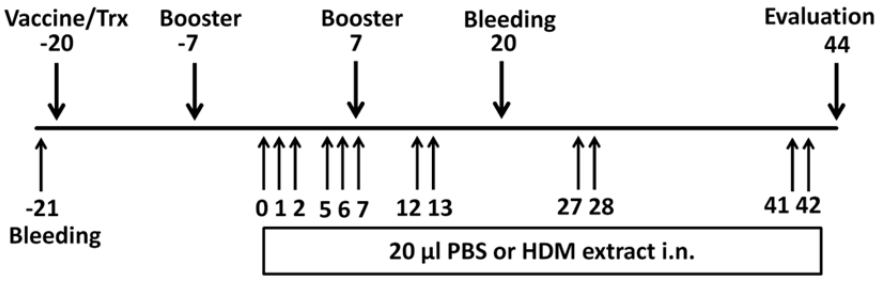

Figure 4. The experimental protocol used in **paper IV**. IL-33 vaccine or control Trx protein were administrated subcutaneously on day -20 and two booster injections on day -7 and 7. The HDM protocol of chronic allergic asthma was initiated on day 0.

2.2.3 BMMC cultures

Bone marrow derived mast cells (BMMCs) were generated *in vitro* by differentiating bone-marrow cells from wild-type C57BL/6 or ST2$^{-/-}$ mice in the presence of IL-3 conditioned medium over a period of five weeks. The purity of mast cells used for lung engraftment of Wsh-mice exceeded 95%.

2.2.4 IL-33 vaccine

Rational design of the IL-33 vaccine construct (Trx-His-IL-33) is depicted in Fig. 5. The vaccine fusion-protein, consisting of the coding region of the *Escherichia.coli* (E.coli) gene for thioredoxin (Trx), His_6- tag and the codon-optimized IL-33 construct (IL-33), was expressed in E.coli. Montanide ISA720 and 1826 phosphorothioate-stabilized CpG oligonucleotide were used as adjuvants.

Figure 5. Schematic structure of the recombinant IL-33 vaccine protein. Three point mutations were introduced in the mouse gene for IL-33 to reduce receptor binding capacity. Trx, thioredoxin; 6His, six-histidine tag.

2.2.5 Lung mechanics

Direct measurements of the respiratory system have been performed using the flexiVent system (Scireq, Montreal, PQ, Canada) and evaluated assuming the constant phase model (paper I-IV), which allows partitioning of lung mechanics into central and peripheral components.

2.2.5.1 The constant phase model

Respiratory input impedance (Zrs) was determined using the forced oscillation technique (FOT), applying pressure oscillations over a range of frequencies. Each determination of Zrs was fit with the constant phase model of impedance [200],

$$Zrs(f) = R_N + i2\pi fI + \frac{G - iH}{(2\pi f)^{\alpha}} \quad (1)$$

Where R_N is the Newtonian resistance closely approximating that of the conducting airways, I is an inertance mostly due to the mass of gas in the central airways, G represents tissue resistance, H characterizes tissue stiffness or elastance, i is the imaginary unit, and f is frequency in Hertz. G and H are linked via the parameter α through the equation:

$$\alpha = \frac{2}{\pi} tan^{-1} \frac{H}{G} \quad (2)$$

Simplified, the constant phase model allows convenient partitioning of the lung mechanics into a central component R_N (reflecting narrowing of the conducting airways) and peripheral components; tissue damping G (closely related to tissue resistance) and tissue elastance H.

2.2.5.2 AHR assessment

To assess AHR, input impedance Zrs was measured in response to incremental doses of acetyl-β-metacholine (Mch) delivered either intravenously (paper **I**, **II**, **IV**) or by aerosol

(paper **III**) and the resultant data was fitted to the constant phase model to obtain the parameters R_N, G and H.

2.2.6 Sample analysis

Differential cell counts [**I-IV**]	Differential cell counts were performed by staining bronchoalveolar lavage (BAL) cytospins or by flow cytometry on single cell suspensions from lung tissue.
ELISA [**I-IV**]	Enzymed linked immunosorbent assay (ELISA) was used to quantify proteins present in BAL fluid (BALF), lung tissue homogenates and serum.
Flow cytometry [**II, III**]	To quantify inflammatory cells in lung tissue, single cell suspensions were stained with fluorochrome-conjugated monoclonal antibodies and analysed on an BD LSRFortessa cytometer. Data was analysed using FlowJo.
Histology [**I-IV**]	Sections from paraffin embedded lung tissue were prepared and stained to detect different structures and immune cells within the tissue. Hematoxylin and eosin staining was used to evaluate inflammatory cell infiltration and smooth muscle cell layer around the airways, periodic acid-Schiff (PAS, Sigma-Aldrich) to assess mucin-containing goblet cells and toluidine blue to quantify mast cells.
Immunohistochemistry [**I**]	Immunohistochemistry staining was performed on lung tissue to assess the presence of IL-33 in the central- and peripheral airways, as well as in the lung parenchyma.
Real-time quantitative PCR [**III**]	Used to measure gene expression of the collagens *Col1a1*, *Col3a1* and *Col5a1* in lung tissue. RNA was extracted from lung tissue, cDNA was synthesized from total RNA and subsequently amplified with specific primer pairs.

2.3 RESULTS AND DISCUSSION

IL-33 and its receptor ST2 have been implicated in several clinical and experimental studies to play important roles in the development of asthma. However, the effect of IL-33/ST2 signaling on airway hyperresponsiveness and inflammation in asthma is not well established and is the focus of study I-IV included in this thesis. In study I, we described how ST2 deficiency affects airway hyperresponsiveness, inflammation and remodeling, all cardinal features of allergic asthma. We then continued by investigating how mast cells contribute to these processes in study II, where ST2 deficiency was limited to mast cells. Study III, explored the potential of IL-33 to exacerbate allergic asthma in sensitized subjects. The effects of IL-33 blocking during the developmental stages of asthma by an IL-33 vaccine was evaluated in study IV.

2.3.1 The interleukin-33 receptor ST2 is important for the development of peripheral airway hyperresponsiveness and inflammation in a house dust mite mouse model of asthma (paper I)

To investigate the role of IL-33/ST2 signaling in promoting asthma development we employed a mouse model of allergic asthma that we established, evaluated and optimized extensively prior to this study. The model is based on intermittent intranasal exposures to HDM (Fig. 2) and has been validated to induce robust chronic inflammatory responses including AHR, airway inflammation, remodeling and antigen-specific IgE production. We chose to evaluate the outcome 48 h after the final HDM exposure, as this was the time point that gave the strongest response for the lung parameters R_N, G and H in a pilot study where airway responses were evaluated on day 1, 2 and 7 after the last HDM exposure. In this study, $ST2^{-/-}$ mice and wild-type BALB/c mice were exposed to HDM extract or PBS as control through intranasal instillations.

Asthma has been characterized as an inflammatory condition affecting first the larger airways and eventually the smaller airways. However, it has also been suggested that the peripheral airway involvement might define a distinct and more severe phenotype of asthma [201]. Since the relative involvement of the central airways versus the peripheral airways has important implications for asthma management [201-203], we thought it is necessary to differentiate between these two compartments when studying pathways involved in asthma development. For this reason, we used the constant-phase model to fit respiratory input impedance, which makes it possible to partition the lung parameters into a central component R_N (reflecting narrowing of the conductive airways), and a peripheral component defined by G and H (reflecting constriction of small airways and alterations in intrinsic lung tissue properties) [204]. We observed that wild-type mice developed a pronounced HDM-induced AHR in both central airways (R_N) and peripheral lung (G and H) relative to the baseline produced by the PBS control mice (Fig. 6). $ST2^{-/-}$ mice developed a similar HDM-induced response in R_N as wild-type mice, indicating that development of AHR in the conducting airways was independent of the IL-33/ST2 pathway. Interestingly, AHR development in the peripheral lung was significantly suppressed in $ST2^{-/-}$ mice as shown by a marked reduction in both G and H. We thereby show distinct effects of HDM-induced ST2 signaling in these two compartments, which suggests that this pathway does not significantly affect the

conductive airways, but instead is important for the development of peripheral changes that contribute to AHR. This distinct effect could be due to distribution of inflammatory cells that express the ST2 receptor and/or that the ability of HDM to induce IL-33 release is greater in the periphery, thereby having a greater impact on responses in this compartment. Other studies have previously linked activation of the IL-33/ST2-pathway with the induction of AHR. Using OVA and ragweed as antigens, AHR was shown to be reduced by blocking antibodies as evaluated by whole-body plethysmograph [35, 205]. Furthermore, reduction in AHR was observed in studies using invasive measurements of lung function in IL-33-deficient mice with ovalbumin as antigen and in neonatal ST2-deficient mice following HDM exposure [11, 206]. However, these studies report only AHR as assessed by overall lung resistance, losing the important information regarding what compartment contributes most significantly to this effect.

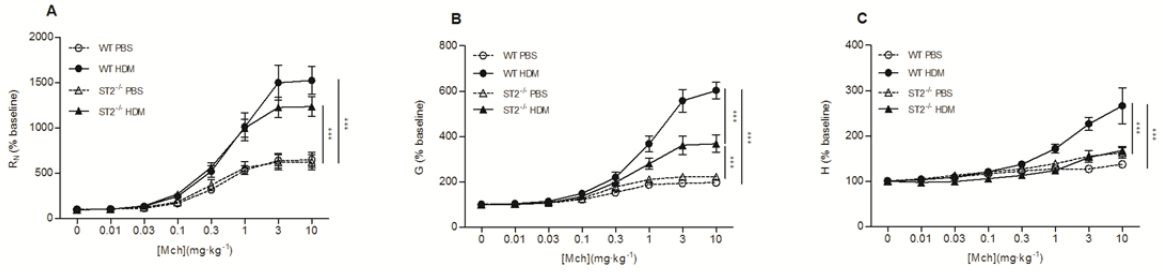

Figure 6. ST2$^{-/-}$ mice show attenuated airway hyperresponsiveness to metacholine in the peripheral lung, as assessed by G and H, compared to wild-type mice. (**a**) Newtonian resistance R_N of the conducting airways, (**b**) tissue damping G, and (**c**) tissue elastance H.

We also observed that ST2 deficiency suppressed pulmonary inflammation and remodeling. Cellular inflammation was assessed in BALF and sections of lung tissue. The number of inflammatory cells in the BALF of ST2$^{-/-}$ mice was considerably lower than in their wild-type counterpart (Fig. 7). The most abundant cells in this model were eosinophils and macrophages.

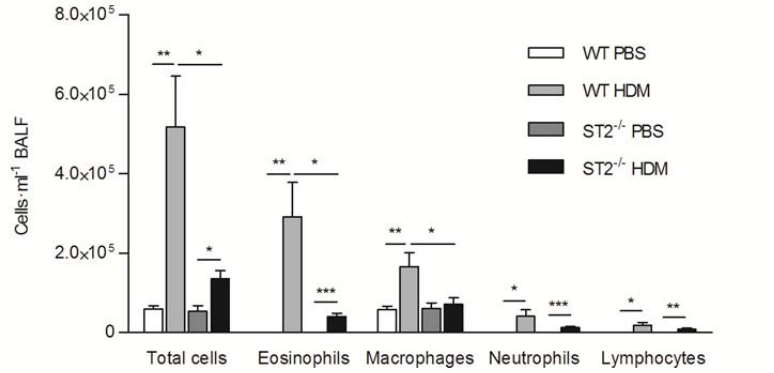

Figure 7. ST2$^{-/-}$ mice have decreased levels of inflammatory cells in bronchoalveolar lavage fluid (BALF) after house dust mite (HDM) exposure compared to wild-type mice. Cellular inflammation, as reflected in the numbers of total and differentiatial cell count of leukocytes in BALF from wild-type and ST2$^{-/-}$ mice treated with HDM extract or PBS. Mean values ± SEM are depicted (n = 7–8 for eachgroup). *P < 0.05, **P < 0.01, ***P < 0.001.

Since we mainly observed effect of ST2-deficiency on peripheral AHR, we wanted to investigate inflammation and remodeling in the peripheral airways in relation to the central airways. We could not observe a significant change in the cellular infiltration around the central airways in lung sections when comparing HDM exposed wild-type and ST2$^{-/-}$ mice.

However, we did find a lower degree of inflammation in the peripheral airways of the HDM exposed ST2$^{-/-}$ mice (Fig. 8a). This effect was also present when evaluating remodeling, where the HDM-induced goblet hyperplasia seen in the wild-type mice was significantly reduced in the peripheral airways of the ST2$^{-/-}$ mice (Fig. 8b).

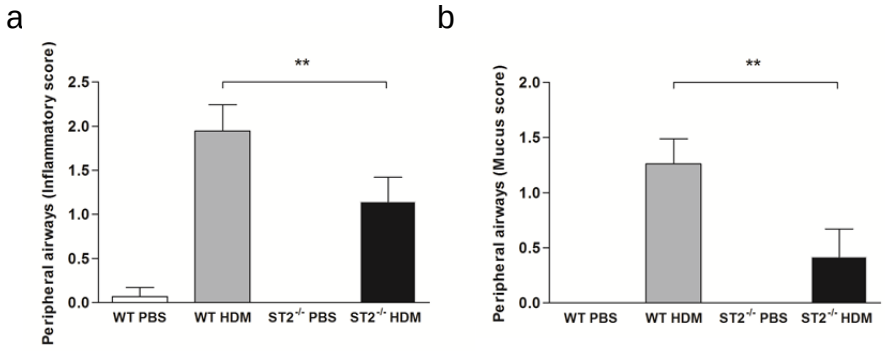

Figure 8. Semiquantitative scoring of the severity of (**a**) inflammatory cell infiltration and (**b**) mucus in the peripheral airways. ST2$^{-/-}$ mice exposed to HDM have diminished inflammation and mucus in the peripheral airways compared to wild-type mice.

ST2 deficiency also reduced the levels of HDM-specific serum IgE. Although, mast cell numbers were not affected, HDM exposure of ST2$^{-/-}$ led to a decrease in the level of the mast cell protease mMCP-1 in BALF. The levels of the HDM-induced inflammatory cytokines including the Th2 cytokines IL-5 and IL-13, Th1 cytokine IL-1β and the epithelial-derrived cytokines IL-33 and GM-CSF were also attenuated in lung homogenates of ST2$^{-/-}$ mice. The decrease of IL-5 and IL-13 may explain the reduced eosinophilia, and goblet cell hyperplasia and AHR, respectively, that is observed in the ST2$^{-/-}$ mice.

When investigating the distribution of IL-33 following HDM exposure in the lung with immunohistochemistry, we observed that the epithelial cell layer in both the central and peripheral airways showed a decrease, whereas the parenchyma showed a marked increase in IL-33. The inflammatory effects in the peripheral lung airways and possibly the subsequent AHR response may be explained by a high induction of IL-33 by alveolar epithelial cells, alveolar machropages and type II alveolar pnemocytes and the presence of IL-33 responsive cells in the peripheral lung compartment [136, 207].

In conclusion, these results suggest a possible asthma phenotype that involves the IL-33/ST2 pathway and is important for the development of airway inflammation and subsequently AHR in the peripheral airways. A feasible explanation for the effect on the peripheral lung function is a combined IL-33 driven increase in peripheral inflammation and mucus production that causes airway closure and thereby increases both parameters G and H.

2.3.2 Mast cell dependent IL-33/ST2 signalling is protective against the development of airway hyperresponsiveness in a house dust mite mouse model of asthma (paper II)

In paper I, we showed that ST2 is important for the development of peripheral airway hyperresponsiveness and lung inflammation in a HDM mouse model of allergic asthma. Based on these findings, we wanted to investigate the role of mast cells in this setting and hypothesized that IL-33/ST2-dependent mast cell responses would contribute to the development of AHR and inflammation.

In order to test this hypothesis, mast cell deficient C57BL/6-Kit$^{W\text{-}sh/W\text{-}sh}$ mice were engrafted with either wild-type (Wsh+MC-WT) or ST2 deficient (Wsh+MC-ST2KO) bone marrow derived mast cells (BMMCs). Allowing 12 weeks for lung engraftment, the mice were subsequently subjected to a HDM protocol (Fig. 2). Mast cell engraftment of the lung compartment was confirmed and was similar in all the groups investigated (Fig. 9). The engraftment was limited to the peripheral lung, predominantly to the alveolar parenchyma, while mast cells were absent around the central airways. This distribution of mast cells in the engrafted Wsh mice is in accordance with previous observations and contrasts to the wild-type situation, in which mast cells can be predominantly found around the central airways [208]. Mast cell localization in humans is more similar to the one found in Wsh mice after mast cell engraftment than to the one in wild-type mice. Therefore, in that aspect, it could be argued that the reconstituted Wsh-mouse is a more relevant model of asthma.

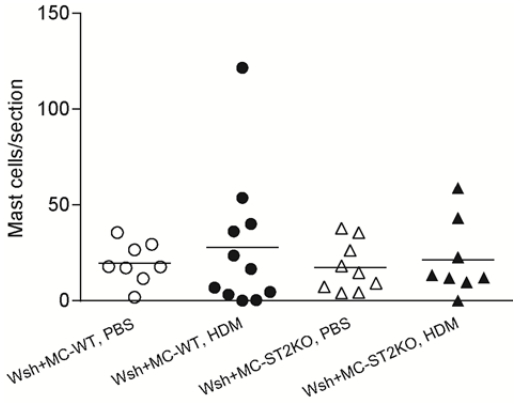

Figure 9. Mast cell deficient C57BL/6-Kit$^{W\text{-}sh/W\text{-}sh}$ mice engrafted with WT or ST2KO BMMCs. Mast cell engraftment of lung tissue evaluated at day 44. Each point represents the mean of mast cell counting from 3-6 lung sections. (Results are expressed as mean of n=8-11 mice in each group.)

Unexpectedly, we discovered an exacerbated development of peripheral AHR in Wsh mice engrafted with ST2 deficient mast cells (Wsh+MC-ST2KO) rather than mast cells with a functional ST2 receptor (Wsh+MC-WT) (Fig. 10). This interesting finding suggests that ST2-dependent mast cell responses can play a protective, rather than causative role in the development of AHR. A protective role for mast cells on airway responses were reported in a study where mast cell deficient mice exhibited exacerbated airway eosinophila in comparison with wild-type mice after inhalation of low-dose papain or low-dose IL-33 [209].

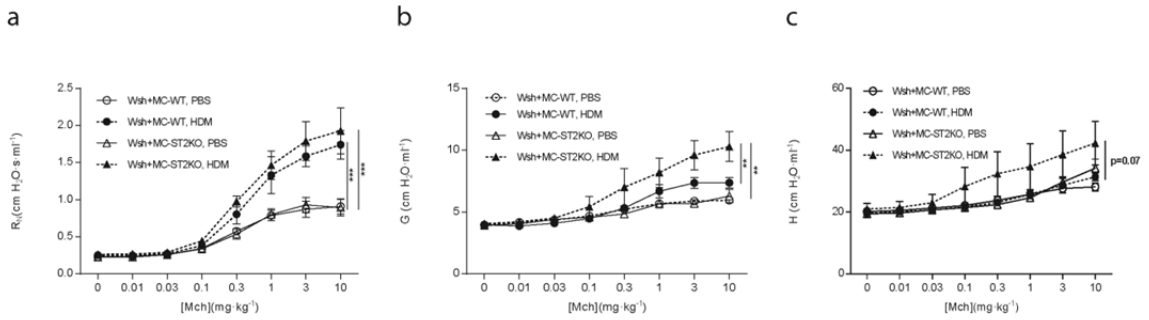

Figure 10. Mast cell dependent IL-33/ST2 signaling protects against the development of airway hyperresponsiveness. Airway hyperresponsiveness (AHR) in response to intravenously delivered methacholine (Mch) was assessed in intubated and ventilated Wsh mice (flexiVent ventilator) engrafted with either wild-type (Wsh+MC-WT) or ST2 deficient (Wsh+MC-ST2KO) mast cells and exposed to HDM extract or PBS. (**a**) Newtonian resistance (R_N) of the central airways, (**b**) tissue damping (G) and (**c**) tissue elastance (H). **p<0.01, ***p<0.001 (ANOVA, Bonferroni). Results are expressed as mean ±SEM of n=8-11 mice in each group.

Next, we investigated how ST2-deficiency in mast cells affected the inflammatory parameters. We did not observe any significant reduction in cellular infiltration in the BALF and lung accompanying the diminished peripheral AHR in HDM exposed Wsh-mice engrafted with mast cells expressing the ST2 receptor (Wsh+MC-WT) compared with those deficient in ST2 (Wsh+MC-ST2KO), which suggests a mechanism independent of the accumulation of inflammatory cells in these compartments. However, the protective effect on AHR was associated with higher levels of PGE_2 in BALF (Fig. 11). A protective role for PGE_2 has been reported previously in asthma patients and inhalation of PGE_2 prevents early and late airway responses after allergen challenge, which can be explained in terms of inhibition of the airway smooth muscle constriction and suppression of mast cell mediators [210].

The protective response on AHR mediated by PGE_2 could thus in this study be explained both in terms of inhibition of the airway smooth muscle constriction and suppression of mast cell mediators or by a yet not identified mechanism. The question remains as to what mechanism mediates the elevated PGE_2 levels in our model. One possibility is that IL-33 induces PGE_2 synthesis and release directly from mast cells or through an intermediate mast cell mediator acting on a PGE_2 releasing target cell. Elevated PGE_2 levels could in turn have a direct relaxing effect on the airway smooth muscle and/or an inhibitory effect on mast cell activity through the PGE_2-mast cell axis subsequently diminishing AHR [211, 212].

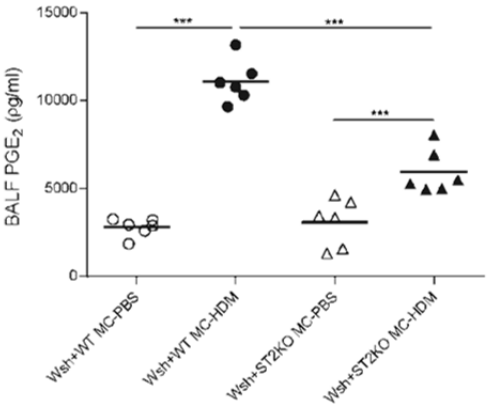

Figure 11. PGE_2 levels were determined in BALF. *p<0.05, **p<0.01 and ***p<0.001 (ANOVA, Bonferroni). Results are expressed as mean ±SEM of n=6-11 mice in each group.

In summary, we have revealed a protective role for IL-33/ST2-dependent mast cell responses in the development of AHR in the peripheral lung that seems to be independent of cellular inflammation yet is associated with elevated PGE_2 levels. More studies are needed to understand the mechanisms underlying the observed effects.

2.3.3 Interleukin 33 exacerbates antigen driven airway hyperresponsiveness, inflammation and remodeling in a mouse model of asthma (paper III)

IL-33 has been identified as a key player in the initiation of asthma pathogenesis. Furthermore, inappropriate and excessive secretion of IL-33 by environmental triggers could exacerbate an unresolved airway inflammation. The most common triggers of asthma exacerbations are respiratory virus infections which are major risk factors for hospital admissions, particularly in combination with allergen exposure in sensitized children acting in synergy to increase the risk of hospital admission [121, 122]. Respiratory viruses and allergens have both been reported to induce IL-33 release from the airway epithelium and dsRNA challenges were reported to produce exacerbation effects in mice with established HDM-induced asthma [213]. With regards to these observations, we hypothesized that IL-33 together with allergen exposure would exacerbate asthma responses in sensitized subjects.

To test our hypothesis, we used a mouse model of asthma in which mice were sensitized to ovalbumin (OVA) (without adjuvant) (Fig. 3). These sensitized mice were subsequently exposed to IL-33 through intranasal instillation before each OVA-challenge. We observed that IL-33 exacerbated many features characteristic of asthma including antigen-induced airway inflammation, AHR and remodeling.

IL-33 administration to OVA-sensitized and challenged mice (OVA+IL-33 group) aggravated antigen-induced AHR in the conductive airways as well as in the peripheral lung, as evaluated by increased R_N, and G and H, respectively in response to increasing doses of methacholine (Fig. 12).

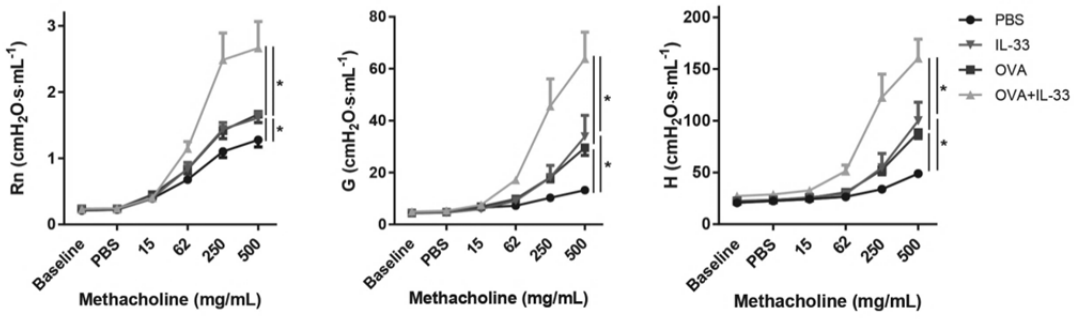

Figure 12. IL-33 potentiates antigen induced airway hyperresponsiveness. Airway hyperresponsiveness (AHR) in response to inhaled methacholine was measured in sham- or OVA-sensitized C57BL/6 mice that received intranasal instillations of IL-33 or PBS. Maximal responses to increasing doses of methacholine are shown for Newtonian resistance (***Rn***), tissue damping (***G***) and tissue elastance (***H***) * $p < 0.05$ (ANOVA, Bonferroni). Results are pooled data from four independent experiments (mean ±SEM of n=9-10 mice for each group).

Exposure to IL-33 alone in mice that were not sensitized and challenged with OVA (IL-33 group), also induced marked increases in R_N, G and H, that were comparable to those in mice that were sensitized and challenged with OVA but not exposed to IL-33 (OVA group). Intranasal administration of IL-33 has previously been shown to induce AHR in BALB/c mice. AHR in response to intranasal OVA instillations to sensitized mice has similarly been observed in another study [214].

We then investigated whether the combined action of IL-33 and OVA exposure in sensitized mice would also enhance airway inflammation. Indeed, the total number of inflammatory

cells and in particular eosinophils in BALF (Fig. 13a), as well as lung tissue inflammation in large airways as well as peripheral lung tissue (Fig. 13b), were severely elevated in the OVA+IL-33 group compared to all the other groups.

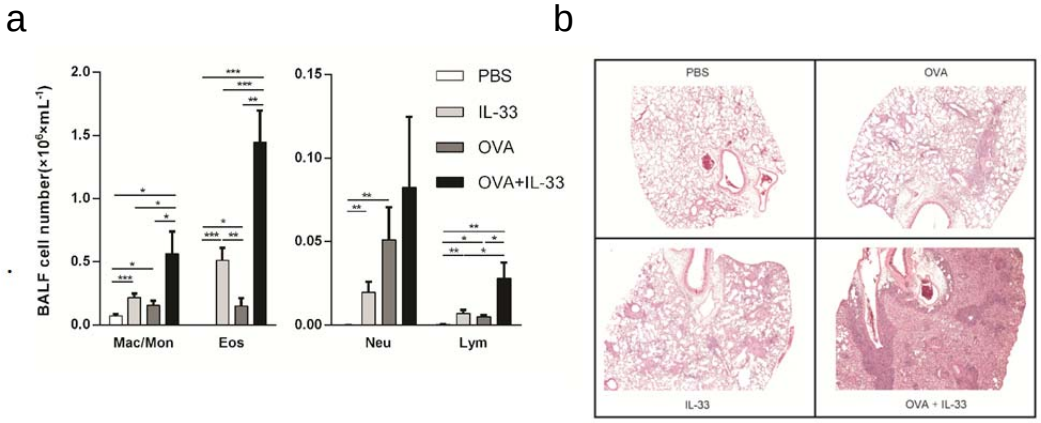

Figure 13. IL-33 potentiates antigen induced inflammation in BALF and the lung tissue. (**a**) Differential cell counts in BALF. (**b**) Representative images of haematoxylin and eosin staining of lung tissue. * p < 0.05, **p<0.01, ***p<0.001 (ANOVA, Bonferroni). Results are pooled data from four independent experiments (mean ±SEM of n=9-10 mice for each group).

Intranasal instillations of IL-33 have previously been shown to induce an increase of eosinophils in murine airways [132].

We could also demonstrate how IL-33 together with OVA-sensitization and challenge affected airway remodeling by measuring the expression levels of three collagen genes; *Col1a1*, *Col3a*1 and *Col5a1* that have been shown to be involved in reticular basement membrane fibrosis in asthma [179], and by assessing the smooth muscle thickness around the central airways. The expression levels of *Col1a1* and *Col3a1* were elevated in the OVA+IL-33 group (Fig. 14a), while IL-33 or OVA alone did not induce expression of the measured collagen genes. Similarly, increase in smooth muscle thickness around the central airways was only increased in the OVA+IL-33 group (Fig. 14b).

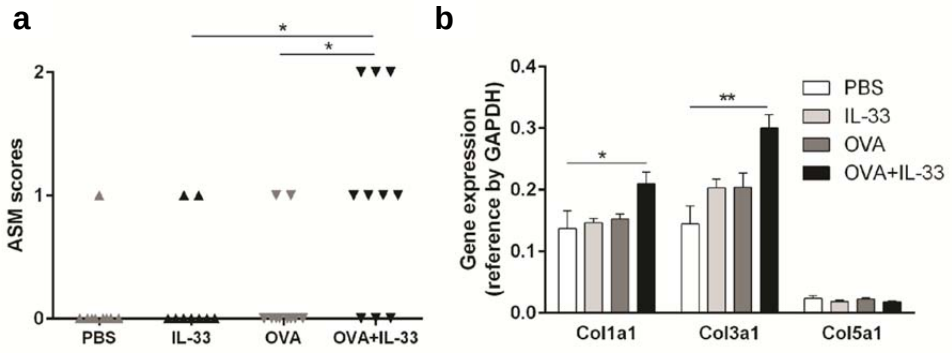

Figure 14. IL-33 together with OVA leads to airway remodeling. (**a**) Scoring of the ASM cell layer around the central airways. The thickness of the ASM cell layer was assessed and a relative score was assigned to each sample, where 0=normal, 1=thickened and 2=substantially thickened ASM cell layer. ASM=airway smooth muscle. *p<0.05 (Chi-square test for trend). Results are pooled data from four independent experiments (mean ±SEM of n=9-10 mice for each group). (**b**) Relative quantitation (RQ) of mRNA levels of *Col1a1*, *Col3a1* and *Col5a1* were assessed in lung specimens. *p<0.05, **p<0.01 (ANOVA, Bonferroni). Results are pooled data from four independent experiments (mean ± SEM of n=3-4 mice in each group).

It has previously been shown that collagen levels in the lung are increased following two weeks of intranasal IL-33 instillations in BALB/c mice [11]. This was not observed in our study in which C57BL/6 mice were exposed to IL-33 for a shorter duration. However, an increase in collagen expression could be observed when IL-33 was combined with OVA. Increased collagen deposition and smooth muscle thickening around the airways reduces the elasticity of the airways and as such, our data suggest that the combined effects of OVA and IL-33 exposure on remodeling of the airways can lead to airflow obstructions.

Taken together we could see that IL-33 exacerbates common features of asthma including AHR, airway inflammation and remodeling. We then wanted to investigate how these observations related to several of the type 2 associated inflammatory responses including antigen-specific IgE production, type 2 cytokine release, mast cell recruitment and activation, and the induction of IL-33 responsive cells including ILC2s.

We could see a striking increase in antigen-specific IgE production induced by IL-33 exposure in OVA sensitized and challenged mice (OVA+IL-33 group) (Fig. 15a), as well as marked increase in the levels of type 2 cytokines IL-4, IL-5 and IL-13, compared to the other groups (Fig. 15b).

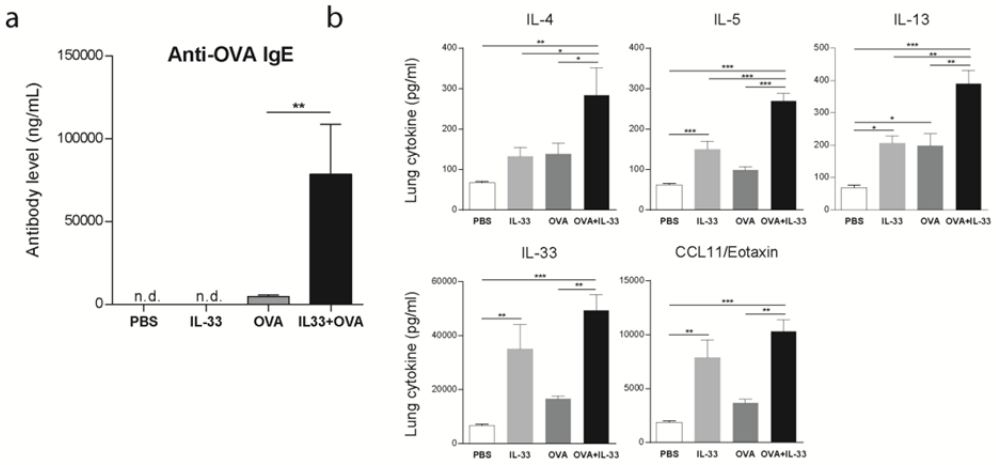

Figure 15. IL-33 exposure enhances antigen induced OVA specific IgE and increases lung cytokine levels in OVA-sensitized mice. (**a**) Serum specimens were analyzed to determine the levels of anti-OVA IgE Abs. n.d.= not detectable. **p<0.01 (ANOVA, Bonferroni). Data are representative of two independent experiments with similar results (mean ±SEM of n=5-6 mice in each group) (**b**) Lung homogenates were analyzed for cytokine levels. *p<0.05, **p<0.01, ***p<0.001 (ANOVA, Bonferroni). Results are pooled data of four independent experiments (mean ±SEM of n=4-6 mice in each group).

It has previously been reported that intraperitoneal IL-33 administration to naïve mice amplified IgE synthesis, independent of antigen, via IL-4 acting on B-cells and that the main source of this cytokine are mast cells and eosinophils [215]. IL-33 enhanced antigen-presentation and activation of naïve $CD4^+$ T cells, and subsequent induction of IgE class-switching in B cells via IL-4, could also explain the enhanced antigen-specific IgE levels. The elevated IL-5 and IL-13 levels could account for the observed IL-33 enhanced airway eosinophilia, and AHR and remodeling, respectively.

When evaluating mast cell density in the lungs, we could not see any significant differences in mast cell expansion in any of the experimental groups. We then hypothesized that the exacerbated airway responses by IL-33 could be explained by an enhancement of mast cell mediator release as opposed to elevated mast cell numbers. Indeed, we could observe elevated levels of the mast cell mediator mMCP-1, which suggests enhanced mast cell activity. This could be due to increased levels of antigen-specific IgE leading to more frequent mast cell degranulation upon antigen encounter or that IL-33 can act directly on sensitized mast cells to increase the storage of mast cell mediators leading to more potent degranulation after IgE cross-linking by antigen. Thus, IL-33 could enhance mast cell responses via an indirect amplification of antigen-specific IgE driven by elevated IL-4 levels as well as directly potentiating mast cell responses by increasing the storage of mast cell mediators.

The increases in type 2 cytokines could be explained by enhanced activity of T_H2 cells, either by a direct action of IL-33 on these $ST2^+$ cells or indirectly through increased activation and differentiation of $CD4^+$ naïve T cells to effector T_H2 cells by IL-33 activated DCs. Another important source of type 2 cytokines, mainly IL-5 and IL-13, are the highly IL-33 responsive ILC2s. ILC2s are innate tissue resident cells that are considered to constitute a potent and earlier source of IL-5 and IL-13. Interestingly, we observed a prominent increase in pulmonary ILC2s, identified as $SSC^{low}FSC^{low}CD45^+Lin^-ICOS^+KLRG1^+Sca\text{-}1^+CD25^+$ $ST2^+$ cells, when OVA-sensitized mice were exposed to IL-33 (Fig. 16), suggesting that ILC2s could be involved in the IL-33 mediated exacerbations of allergen-induced asthmatic responses presented herein.

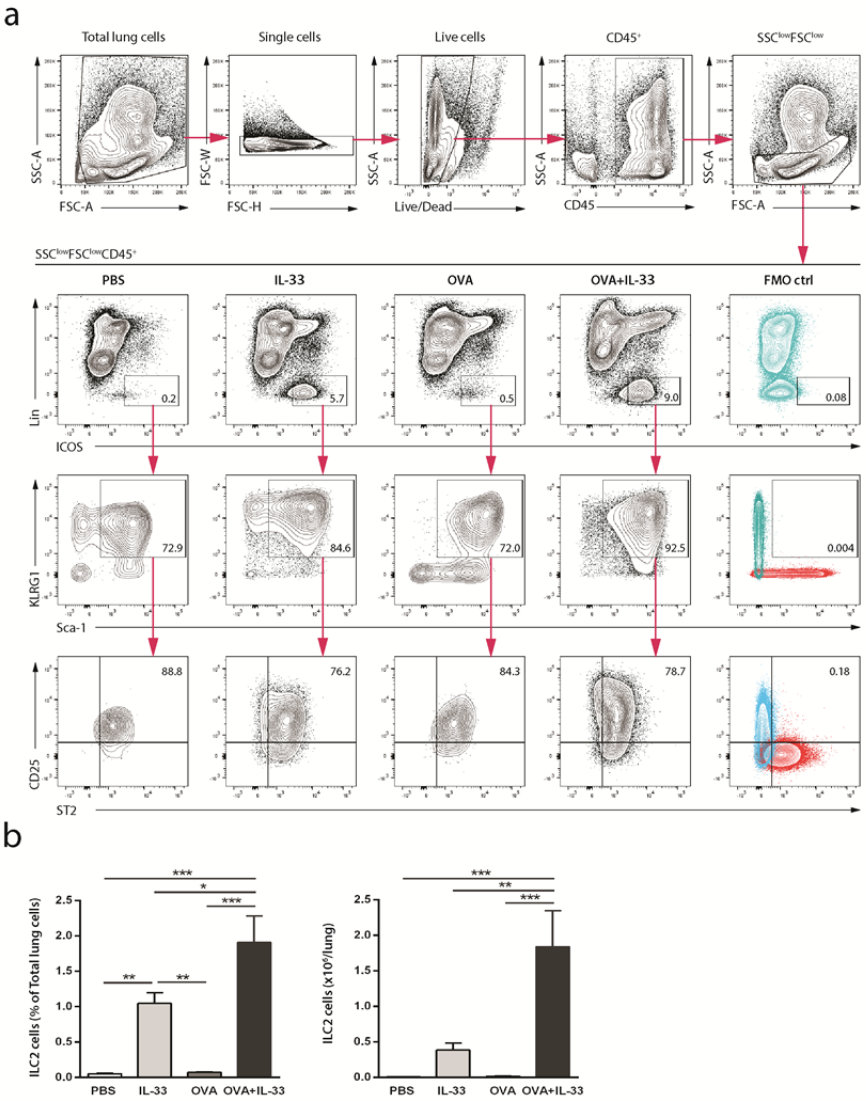

Figure 16. IL-33 enhances the accumulation of pulmonary ILC2s in OVA-sensitized mice. Flow cytometric analysis of lung cells. (**a**) ILC2s were identified as $SSC^{low}FSC^{low}CD45^{+}Lin^{-}ICOS^{+}KLRG1^{+}Sca\text{-}1^{+}CD25^{+}ST2^{+}$ cells. Gates were set based on FMO controls, FMO ctrl= overlay of the two FMO controls for each respective plot. The representative FMO plots and plots showing the representative $SSC^{low}FSC^{low}CD45^{+}$ gating belong to the OVA+IL-33 group. (**b**) Frequency and numbers of pulmonary ILC2s. *p<0.05, **p<0.01, ***p<0.001 (ANOVA, Bonferroni). Results are pooled data of four independent experiments (mean ±SEM of n=8 mice in each group).

In conclusion, this study provides evidence that IL-33 exacerbates antigen-driven features of allergic asthma and we propose that mast cells and ILC2s have a central role in driving these processes. It remains elusive whether the observations we report herein can be coupled to the exacerbation of asthma symptoms observed in sensitized individuals. Exacerbations of allergic asthma could be explained by an inappropriate and excessive release of IL-33 caused by epithelial triggers such as viral infection. Collectively, our data suggests that IL-33 constitutes a potential target for the management and prevention of asthma exacerbations.

2.3.4 Vaccination against IL-33 inhibits airway hyperresponsiveness and inflammation in a house dust mite model of asthma (paper IV)

In paper IV, we evaluated a vaccination strategy that targets IL-33. The rationale behind the design of this vaccine was analogous with vaccination strategies for preventing infectious disease; to induce immunological memory responses to an antigen. However, vaccination against endogenous antigens such as IL-33 poses a problem with regard to self-tolerance. In order to overcome this, the self-antigen must be modified by insertion of dominant T cell epitopes or linking it to a non-self-antigen [216]. Here, the recombinant IL-33 was coupled to a small redox protein called thioredoxin derived from E.coli creating a recombinant fusion protein (Fig. 5). Furthermore, three point mutations were introduced to the recombinant IL-33 protein with the purpose to reduce receptor binding capacity, while retaining most of its B cell epitopes. The modified IL-33 vaccine protein was used together with the adjuvants Montanide ISA 720 and phosphorothioate stabilized CpG oligonucleotide 1826, which have previously been shown to be effective in vaccination against endogenous antigens [217-220].

The efficacy of this vaccine was evaluated in our mouse model of HDM-induced asthma using a prophylactic strategy where female BALB/c mice were immunized subcutaneously with the IL-33 vaccine or the carrier protein (Trx) with the adjuvants as control (Fig. 4).

First, we tested whether the IL-33 vaccine was able to elicit a humoral response with the production of blocking IgG-antibodies against IL-33. The presence of anti-IL-33 IgG was determined using ELISA and revealed that high titers of anti-IL-33 antibodies were obtained on days 20 and 44 in vaccinated mice (Fig. 17). These observations suggested that the recombinant IL-33 vaccine was immunogenic. We then wanted to investigate whether the induction of IL-33 blocking antibodies could ameliorate the effect of HDM-induced airway responses.

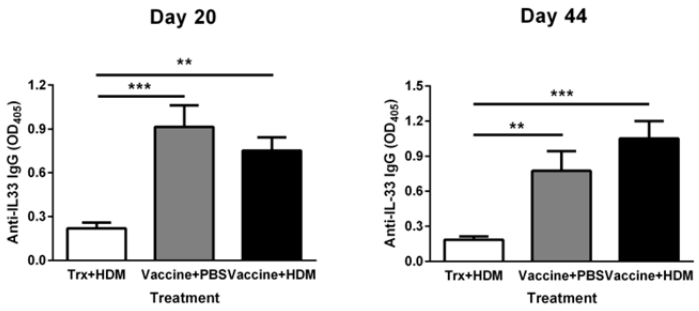

Figure 17. IL-33 vaccine elicits anti-IL33 specific antibodies. Serum samples of anti-IL-33 IgG on days 20 and days 44. All values shown are means ± s.e.m (n = 10 mice in each group on day 20; n = 9 mice in HDM+Vaccine group, n = 10 in PBS+Vaccine group and n = 10 in Trx+HDM group on day 44, ** P < 0.01, *** P < 0.001 in comparison to the Trx + HDM group. Vacc: IL-33 vaccine, Trx: thioredoxin, HDM: housedust mite, N.D.: not detected.

By evaluating AHR in response to increasing doses of MCh we observed that the HDM-induced (HDM+Trx) resistance in the conducting airways R_N was attenuated by approximately 50% and tissue resistance G was completely abolished by the IL-33-vaccine (HDM+vaccine), indicating an IL-33 dependent effect on both conductive and distal airways in this model (Fig. 18).

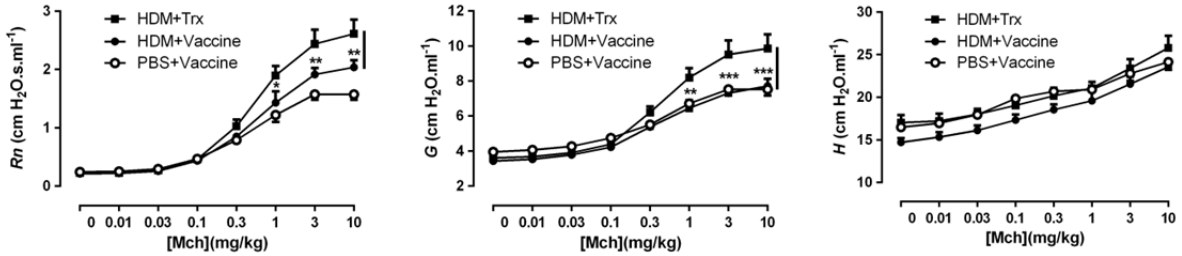

Figure 18. Vaccination against IL-33 diminishes house dust mite-induced airway hyperresponsiveness to methacholine. The resistance of the central airways (Rn), pulmonary tissue damping (G) and tissue elastance (H) to methacholine (Mch) were assessed employing a flexiVent. All values shown are mean ± s.e.m. (n = 8 mice in HDM+Vaccine group, n = 9 mice in Trx+HDM group and n = 6 mice in PBS+Vaccine group, missing samples due to unsuccessful or uncompleted intravenous injection in the animals) *P < 0.05, ** P < 0.01 in comparison to the Trx+HDM group. Vacc: IL-33 vaccine, Trx:thioredoxin, HDM: house dust mite.

When we investigated the effect of IL-33 vaccination on inflammation we discovered that the HDM evoked inflammation (HDM+Trx) was suppressed by the IL-33 vaccine (HDM+vaccine) as eosinophilia was considerably diminished in BALF (Fig. 19a), and lung tissue inflammation was reduced (Fig. 19b). This finding is in agreement with previous studies reporting that blocking IL-33 with mAbs attenuated OVA-induced eosinophil infiltration [221, 222]. We also observed that the IL-33 vaccine attenuated the HDM-induced levels of the inflammatory cytokines including IL-25, IL-33, TSLP, and IL17A, but did not have an effect on the levels of IL-5 and IL-13. A possible explanation for the absence of an effect on IL-5 and IL-13 is that we could have missed the window where these cytokines peak, which has been reported to occur four hours after the final HDM exposure [223].

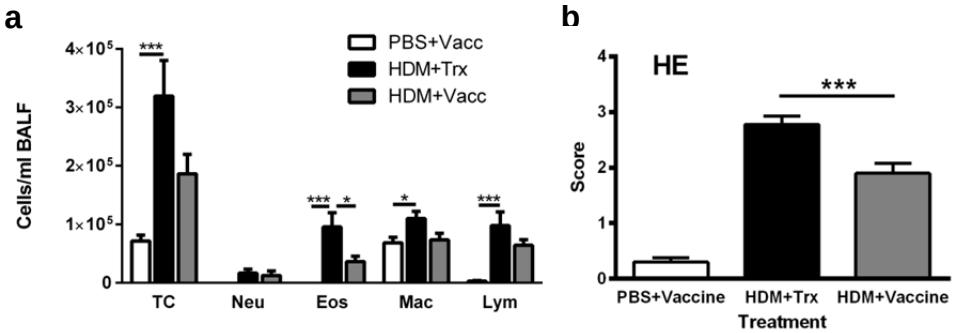

Figure 19. Vaccination of mice against IL-33 attenuates the increase in the number of inflammatory cells present in bronchoalveolar lavage fluids and pulmonary tissue inflammation. (**a**)Total and differential cell counts were performed on cytospin preparations of the BALF. (**b**) Semi-quantitative scoring of hematoxylin and eosin (H&E) staining. All values shown are mean ± s.e.m. (n = 9 mice in HDM+Vaccine group, n = 10 mice in PBS+Vaccine group and n = 10 mice in Trx+HDM group, missing sample due to failed collection of BALF from the animal). * P < 0.05 in comparison to the Trx+HDM group. Vacc: IL-33 vaccine, Trx: thioredoxin, HDM: house dust mite, TC: total cell count, neu: neutrophils, eos: eosinophils, mac:macrophages, lym: lymphocytes.

In summary, we have shown that a novel IL-33 vaccine elicits high titers of antibodies to IL-33 that are sustained and significantly inhibit HDM-induced AHR, airway inflammation and production of pro-inflammatory cytokines. These observations lend further support to the proposal that IL-33 plays a key role in the initial pathogenesis of asthma [28]. Thus, the IL-33 vaccine can serve as a tool to elucidate the role of IL-33 in allergen-induced asthma responses and as our results suggest could be further evaluated for therapeutic purposes. Exacerbations of asthma might in part be driven by IL-33, as suggested by the results in study III, thus blocking IL-33 could also prove to be an effective therapeutic tool for management and prevention of asthma exacerbations in addition to treatment of asthma phenotypes where IL-33 is implicated [224].

2.4 CONCLUSIONS

The main conclusions from this thesis are:

Paper I: The IL-33/ST2 pathway contributes to the development of several characteristic features of asthma, including AHR, inflammation and remodeling. Importantly, this study shows that the ST2-dependent contribution to AHR mainly resides within the peripheral lung compartment. We suggest that this effect is driven by peripheral inflammation and airway remodeling that causes airway closure. Thus, the IL-33/ST2 pathway may constitute an important target in the treatment of small airway disease.

Paper II: Mast cell responses can have a protective role in the development of AHR by constraining the deleterious IL-33/ST2 mediated responses. This study also supports the notion that AHR can be an event occurring independent of airway inflammation. We suggest that ST2 dependent mast cell responses attenuate AHR at least in part through the induction of bronchoprotective PGE_2.

Paper III: IL-33 exacerbates allergen-induced airway responses including AHR, inflammation and remodeling. Particularly, IL-33 exposure following antigen sensitization enhances AHR in the conductive airways as well as in the peripheral lung compartment, boosts antigen-specific IgE production, and leads to the expansion of $ST2^+$ ILC2s. We conclude that since exacerbations of allergic asthma could be explained by an inappropriate and excessive release of IL-33 caused by epithelial triggers such as viral infections, IL-33 constitutes a potential target for the management and prevention of asthma exacerbations.

Paper IV: The development of asthma features including AHR, airway inflammation and remodeling can be at least in part prevented by a vaccination strategy using a recombinant IL-33 protein that induces the production of IL-33 blocking IgG antibodies. These observations further support the key role of IL-33 in the establishment of asthma. Furthermore, since IL-33 likely contributes to asthma exacerbation, this vaccine strategy represents a potential therapeutic tool for the prevention and treatment of asthma exacerbations.

2.5 FINAL REFLECTIONS AND FUTURE PERSPECTIVES

This thesis investigated the role of the IL-33/ST2-pathway in mouse models of allergen-induced asthma. The knowledge gained from the work in this thesis provides further evidence and new insights into the importance of this pathway in asthma. In this section, I present some of my reflections and speculations regarding the research field related to the IL-33/ST2-pathway in asthma, which would be interesting to address in the future.

The origins of asthma remains enigmatic but is considered to be caused by environmental triggers in genetically predisposed individuals. Only a few genes have consistently been associated to asthma. Two of the most replicated candidate genes for asthma from large genome-wide association studies (GWAS) include the genes for IL-33 (*IL33*) and ST2 (*IL1RL1*). Single nucleotide polymorphisms (SNPs) in and flanking these genes are thus associated with increased susceptibility to asthma. It is interesting to consider how these genetic variants translate into consequences for protein expression, structure, and ultimately the activity of the IL-33/ST2-pathway. At present, this knowledge is very limited. However, SNPs can in general be translated into functional alterations by two main mechanisms; through changes in the level of expression or through amino acid substitutions that can alter protein structure and function. Most of the *IL33* associated SNPs are located 5' of the gene or in the first intron, while some *IL1RL1* associated SNPs are also located in exons [225]. These observations suggest that IL-33 activity could mainly be affected by increased expression, while ST2 could also be associated with structural and functional alterations of the protein. Interestingly, a recent study identified a loss-of-function sequence variant in the gene for *IL33* that resulted in reduced expression and a truncated form of IL-33 that lacked cytokine activity due to an inability to bind to the ST2 receptor, and as a consequence was associated with attenuated inflammatory responses leading to reduced eosinophil counts [126]. It is tempting to speculate that other genetic variants of *IL33* and *IL1RL1* exist that could result in structural alterations that instead potentiates their activity. It would be of great interest to identify the structural and functional consequences for these gene variants.

In paper III, we showed that exposure of sensitized mice to IL-33 exacerbated allergen-induced AHR, inflammation and remodeling. This approach can be seen as a way to mimic asthma exacerbations as a consequence of insults such as virus infections that lead to IL-33 release or it could also be seen as a way to simulate an excessive IL-33 release caused by a genetic variant. However, it does not address the effects of a functionally different IL-33. Under the assumption that genetic variants of IL-33 leading to structural alterations could be identified and expressed as recombinant proteins, it would be very interesting to investigate their effects in our model. Similarly to the more biologically active splice variants of IL-33 created after cleavage by inflammatory proteases from mast cells and neutrophils [158, 159], genetic variants of IL-33 resulting in a truncated or alternatively folded protein could lead to enhanced biological activity. There are several ways through which enhanced biological activity could be conferred; higher affinity of IL-33 to the ST2 receptor, enhanced stability of IL-33, unresponsiveness to negative regulation such as sST2 binding and proteolytic inactivation of IL-33, impaired retention capability in the cell nucleus resulting in aberrant release, and altered ability to recruit accessory proteins to the IL-33R complex after IL-33

binding. The latter is especially compelling, since it could lead to amplified signaling transduction, such as the one that exist in mast cells where KIT is recruited to the heterodimer that is formed by ST2 and its accessory receptor IL-1RAcP after IL-33 binding, which leads to a synergistic amplification of IL-33 signaling [155]. In addition to amplified signaling, such a possibility also implies that different IL-33 variants could selectively activate different cells, depending on their expression of co-receptors and accessory proteins. The expression of KIT in non-neoplastic cells is restricted to hematopoietic stem cells, progenitor cells, mast cells and ILC2s. Different IL-33R complexes could be important in other cell types. Extending on this thought; different IL-33 variants could induce different inflammatory profiles by the activation of different immune cells, and perhaps these profiles could be translated into asthma subtypes i.e. different IL-33 variants may be of distinctive importance depending on asthma subtype.

Additionally, the presence of IL-33 protein variants could have important implications for therapeutic approaches targeting IL-33, as IL-33 inhibitors need to target all biologically active IL-33 variants. Similarly, this needs to be taken into consideration when developing anti-IL-33 antibodies used in various assays for measuring IL-33 levels in patients. In study IV, we evaluated the potential of an IL-33 vaccine for preventing the development of asthma in a mouse model. This strategy proved effective in our model, in which IL-33 specific IgG antibodies were induced that attenuated AHR and inflammation. Vaccination against IL-33 opens an interesting possibility in the context of this discussion. Perhaps the IL-33 antigen used for vaccination could be modified to selectively induce blocking antibodies to more effectively target specific IL-33 variants.

In addition to the functional consequences of *IL33* variants, little is known about how IL-33 release is regulated. It is relatively well established that insults resulting in necrotic cell death leads to a passive release of IL-33 from the nucleus [146]. Less is known about the triggers that result in active IL-33 release, although it is evidently possible as illustrated by studies that show IL-33 release without any apparent cell death [150, 151]. What further adds to the enigma is how IL-33 secretion can occur in the first place as IL-33 is translated without a signal sequence for secretion via the classical-pathway (endoplasmic reticulum/golgi pathway). It is possible that IL-33 release can be mediated by different non-classical pathways involving other types of secretory vesicles such as exosomes [153].

It is tempting to speculate that polymorphism in genes for effector molecules involved in regulating the release and activity of IL-33 are of equal importance. I believe that insights into the mechanisms of IL-33 release regulation during different developmental stages in response to environmental factors are of key value in understanding the pathogenesis of asthma. In this scenario, events upstream of IL-33 release are of critical value as these could constitute potential targets to prevent aberrant IL-33 release from occurring in the first place. Furthermore, they could provide more selective targeting of IL-33 under the assumption of a more specialized regulation of IL-33 release by specific triggers. This selective targeting would be particularly desirable considering the protective role of IL-33 in several diseases including cardiovascular diseases [148, 187, 188].

An interesting aspect is the interaction of environmental stimuli such as allergens and viruses with epithelial pattern recognition receptors (PRRs) and the possibility of these interactions in IL-33 release regulation. Exposure to the house dust mite allergen with proteolytic activity has been shown to trigger TLR4 on the pulmonary epithelial cells resulting in the production of IL-33 [150]. As such, the ability of allergens to trigger IL-33 release via PRRs could at least in part account for their allergenicity. Furthermore, given the importance of epithelial PRRs in promoting IL-33 release, therapeutic modulation of PRRs could also prove beneficial and more specific than targeting IL-33 directly.

In addition to IL-33 release mediated by PRRs, it is interesting to speculate that active release of IL-33 in asthma could be regulated by structural factors involved in maintaining barrier integrity of the airway epithelium. As a result, defects in these proteins could lead to an inappropriate IL-33 release when these barriers are breached for instance by cleavage of tight-junction molecules by allergens with proteolytic activity such as the house dust mite allergen Der p 1 [226]. An interesting scenario where epithelial contacts are broken is during physiological airway remodeling that involves structural changes that occur during normal lung development. In this setting, improper IL-33 activity could turn a physiological process into a pathological one. In a recently published study it was found that IL-33 is spontaneously produced during the alveolar phase of lung development in mice, which led to an spontaneous accumulation of type 2 cells including ILC2s, eosinophil, mast cells and basophils shortly after birth [227]. HDM exposure of mice at this developmental stage further increased IL-33 and enhanced Th2 skewing, compared to adult mice. These results could explain why children under 3 years, at a time when their lungs are still developing, are at a high risk of developing allergic disease to inhaled allergens and how some viral infections at this age could exacerbate this process even further. It is tempting to speculate that an impaired IL-33 release at this stage could lead to altered remodeling during the alveolarization phase taking place in the distal lung and perhaps explain why airway remodeling is already observed in preschool children even in the absence of inflammation [103] and support the notion that airway remodeling, rather than a secondary event to inflammation, can evolve as a primary event initiated early in life [102].

In study III, we performed a transcriptome analysis of the lung tissue, which showed up-regulated expression of several genes in the combination group of IL-33 and OVA (data not presented in the paper) including significant up-regulation of genes for proteins that are involved in stress and wound repair of the barrier epithelia. It would be interesting to follow up on this finding and see if these genes are alternatively regulated in epithelial cells derived from asthma patients and their consequences for IL-33 release. Also, it would be interesting to investigate the expression of different PRRs on the surface of epithelial cells from asthma patients and the effect they have on IL-33 release in response to different triggers such as allergens and virus-antigens.

Although, the focus of this thesis has been on the impact of IL-33 to exacerbate type 2 inflammatory processes, it is now accepted that IL-33 also contributes to other immune responses such as T_H1 and T_H17 responses [228]. Interestingly, a mixed T_H1 and T_H17 cytokine profile is characteristic of a subset of asthma patients with late-onset and more

severe forms of asthma with less reversible airway obstructions [12-14]. In the future, it would therefore be of interest to further understand the role of IL-33 in activing other immune responses in relation to different asthma subtypes. In study II we identified a protective role for IL-33/ST2 dependent mast cell responses on AHR development, which suggests that IL-33 can have different roles even in the same disease. Thus, the context in which IL-33 is present is important for the kind of immune response triggered. It is thus essential to understand under which conditions IL-33 contributes to a pathological response over a protective one, especially when considering IL-33 as a candidate for therapeutic interventions.

In conclusion, there is a need for a better understanding of how *IL33* and *IL1RL1* polymorphism translates into consequences for structure and function of their respective proteins. Furthermore, it is important to identify the mechanisms that regulate IL-33 release, including the interaction of different allergens and viral antigens with various PRRs, as well as factors important in maintaining epithelial integrity, to enable more specific targeting of this pathway. Also, the ability of IL-33 in activating other immune responses than type 2 needs to be further characterized in relation to different asthma subtypes.

3 POPULÄRVETENSKAPLIG SAMMANFATTNING

Astma är ett samlingsnamn för olika sjukdomar i luftvägarna med gemensamma kännetecken som inflammation, lättirriterade luftvägar som tenderar att dra ihop sig, samt strukturförändringar som påverkar lungans normala funktion. Detta innebär i sin tur att luftflödet till lungorna kan begränsas, vilket kan ge upphov till symptom i form av hosta, pipande och väsande andning, samt andnöd. Symptomen återkommer i episoder och kan förvärras av luftvägsinfektioner, framför allt orsakade av virus, samt vid exponering för allergiframkallande ämnen.

Mellan åtta och tio procent av Sveriges befolkning lider av någon form av astma. Det finns många olika typer av astma som orsakas av olika anledningar. Den vanligaste formen av astma är allergisk astma som utlöses om man kommer i kontakt med ämnen man inte tål, så kallade allergener, vanligtvis pollen, pälsdjur eller kvalster. Kroppens immunförsvar sätter då igång en inflammatorisk reaktion som på kort sikt leder till en sammandragning av luftvägarna och en ökad slemproduktion, men kan även i ett längre perspektiv leda till kroniska skador på luftvägarnas struktur med försämrad lungfunktion som följd.

Orsaken till astma är inte klarlagd men både arv och miljö och i synnerhet samspelet mellan arv och miljö har stor betydelse för risken att drabbas av astma. Risken för att drabbas av astma ökar således om man bär på vissa gener. Det är viktigt att identifiera dessa gener och förstå hur de påverkar uppkomsten av astma för att kunna utveckla bättre behandlingsformer som i dagsläget endast behandlar symptomen och inte är tillräckligt effektiva hos ett stort antal individer med astma. Flera studier har under senare år visat att det finns ett samband mellan genen för ett signaleringsprotein, kallat IL-33, och utveckling och svårighetsgrad av astma. Detta tyder på att IL-33 kan utgöra en möjlig kandidat för behandling av astma, men först behöver man öka förståelsen för dess funktion och hur den påverkar uppkomsten och utvecklingen av astma. IL-33 är ett protein som finns framför allt i epitelceller som bildar en skyddande barriär mot omvärlden i lungan. När epitelcellerna kommer i kontakt med smittämnen, allergen eller andra irriterande ämnen som ger upphov till skada, släpper epitelcellerna ut IL-33 som i sin tur signalerar till omgivande immunceller som kan känna av IL-33 genom en receptor som finns på cellens yta, kallad ST2, att en skada har uppstått. Detta leder i sin tur till en inflammatorisk reaktion med syfte att neutralisera det främmande ämnet och reparera skadan. I vanliga fall är detta ett önskvärt förlopp, då kroppen reagerar med en inflammation för att försvara sig mot olika smittämnen, men hos individer med astma kan ett felaktigt utsläpp av IL-33 leda till en okontrollerad inflammatorisk reaktion som kan bli bestående och leda till förändringar i luftvägarnas struktur och funktion.

Den här avhandlingen består av fyra delarbeten som syftar till att förstå hur IL-33 bidrar till uppkomsten och utvecklingen av astma, samt undersöker om man genom att blockera IL-33 med ett vaccin kan förhindra att astma uppstår. För att undersöka detta har vi använt oss av musmodeller i alla fyra delarbeten. Musmodellerna har vissa begränsningar eftersom möss inte spontant utvecklar astma, men genom att utsätta dem upprepade gånger för olika allergen kan man framkalla ett astmaliknande tillstånd som har många av de kännetecken som finns

hos människan. En stor fördel med musmodeller är att man hos möss kan slå ut gener och således studera vilken betydelse avsaknadet av en viss gen har för sjukdomsförloppet. Det finns också tillgång till många verktyg som gör det möjligt att mäta viktiga faktorer som styr sjukdomsförloppet.

I den första studien undersökte vi hur viktig IL-33 är för utveckling av astma genom att använda oss av möss som saknar ST2 (receptorn för IL-33), och därmed även möjligheten att svara på IL-33, samt normala möss med en fungerande ST2 receptor som kan känna av IL-33. De här mössen utsattes sedan för kvalsterallergen i syfte att framkalla astma och vi kunde sedan studera hur olika reaktioner skiljde sig mellan dessa två grupper. Vi kunde bland annat se att möss som saknade ST2 inte utvecklade lika omfattande överkänslighet i de små luftvägarna som möss med ST2, medan överkänsligheten i de stora luftvägarna inte skiljde sig åt i de två grupperna. Detta betyder att IL-33 är viktigt för att framkalla överkänslighet hos de små luftvägarna men påverkar inte nämnvärt överkänsligheten hos de stora luftvägarna. Det är ett viktigt fynd då de små luftvägarna är inblandade i svårbehandlad astma och hämning av IL-33 skulle i dessa fall kunna vara en effektiv behandlingsform. Vi kunde även visa att den inflammatoriska reaktionen samt vävnadsförändringarna i de små luftvägarna dämpades hos möss som saknade ST2. Tillsammans visar dessa fynd att IL-33 är viktig vid uppkomsten av ett flertal reaktioner som är centrala i astmautveckling.

I den andra studien undersökte vi hur mastceller, genom att svara på IL-33, bidrar till utveckling av överkänslighet och inflammation hos luftvägarna i samma musmodell som i den första studien. Mastceller är en typ av inflammatoriska celler som är inblandade i allergiska reaktioner och är således viktiga vid allergisk astma. När en person som bär på anlag för att utveckla allergisk astma mot exempelvis pollen kommer för första gången i kontakt med pollen, bildar kroppens immunförsvar så kallade IgE antikroppar som kan känna igen olika delar av pollen. Den här processen kallas för sensibilisering varvid mastcellen förbereder sig inför nästa pollenexponering genom att binda dessa antikroppar till sin yta. Nästa gång personen utsätts för pollen, kommer IgE antikropparna på mastcellens yta således känna igen pollen och reagera genom att utsöndra substanser som påverkar luftvägarnas förmåga att dra ihop sig och kan sätta igång många andra inflammatoriska reaktioner. Det är även känt att mastceller kan påverkas av IL-33, men det är inte helt klarlagt hur mastceller genom att känna av IL-33 bidrar till utveckling av luftvägsresponser som är centrala i astmautveckling. För att studera detta använde vi i den här studien möss som saknade ST2 receptorn på endast mastceller, till skillnad från den första studien då alla celler saknade ST2, vilket möjliggör utredning av effekter när mastcellerna inte kan känna igen IL-33. Ett viktigt fynd i den här studien var att mastcellerna visade sig ha en skyddande roll i utveckling av luftvägsreaktivitet som svar på IL-33 och vi visar att den effekten har ett samband med förhöjda nivåer på ett ämne som kallas PGE_2 som i tidigare studier har visat skydda luftvägarna från att dra ihop sig. Sammantaget, tyder resultaten från de båda studierna att IL-33 kan ha både en skadlig och skyddande roll i utvecklingen av astma.

I den tredje studien ville vi undersöka hur luftvägsreaktivitet, inflammation och vävnadsförändringar i luftvägarna påverkas om man utsätter sensibiliserade möss (möss som

redan bildat IgE antikroppar och är därmed rustade för återkommande exponering för allergenet), för det allergen de är sensibiliserade mot i kombination med IL-33. Det här scenariot kan ses som en förenklad modell av hur en ökad frisättning av IL-33 på grund av exempelvis virusinfektioner skulle kunna förvärra (exacerbera) astmasymptom hos personer med allergisk astma. Det visade sig att IL-33 tillsammans med allergenet förvärrade astmatillståndet inklusive luftvägskänsligheten, inflammationen samt luftvägsförändringar. Det här fyndet är av betydelse då en överdriven frisättning av IL-33 hos personer med allergisk astma skulle kunna förklara varför deras symptom förvärras vid exempelvis virusinfektioner. En annan konsekvens av fynden i den här studien är att man skulle kunna blockera IL-33 för att behandla exacerbationer av astma.

I den fjärde och sista studien utvärderade vi om man med hjälp av ett vaccin mot IL-33 kan i likhet med vaccination mot smittämnen inducera ett immunologiskt svar som leder till att antikroppar som blockerar IL-33 kan bildas. Till skillnad från vacciner som riktar sig mot smittämnen, så medför vaccination mot ett kroppseget ämne som IL-33 en utmaning då kroppens immunförsvar är tränat till att inte svara på kroppsegna ämnen. Vaccinet måste således framställas så att immunförsvaret kan känna igen det och samtidigt inte ge upphov till skadliga effekter. För att uppnå detta har vi i den här studien förändrat strukturen på vaccinet så att kroppens immunförsvar kan bilda antikroppar mot IL-33 utan att sätta igång en skadlig inflammation. Det här vaccinet gavs sedan till möss innan de utsattes för kvalsterallergen och ytterligare påfyllnadsdos gavs efter påbörjad allergenexponering. Vi fann att IL-33 vaccinet både förhindrade luftvägsreaktiviteten och sänkte det inflammatoriska svaret genom att bilda antikroppar mot IL-33. Resultaten tyder således på att vaccinering mot IL-33 kan vara ett effektivt sätt att förhindra uppkomsten av astma samt användas vid behandling av astmaexacerbationer.

Sammanfattningsvis ger våra studier stöd för att IL-33 har en viktig roll vid uppkomsten av astma och ger effekter framför allt i de små luftvägarna. Studierna tyder även på att IL-33 kan förvärra astmatillståndet hos en person med allergisk astma samt att behandling som motverkar effekten av IL-33 skulle kunna vara ett effektivt sätt att förhindra både uppkomsten av astma och dämpa astmaexacerbationer hos personer med astma. Samtidigt är det viktigt att i fortsatta studier utvärdera hur resultaten förhåller sig till människan.

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